APPLICATION SOLUTIONS FOR METABOLITE IDENTIFICATION

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ADDRESSING DRUG METABOLISM IN A CHANGING PHARMACEUTICAL INDUSTRY

Metabolic determinations are an integral part of every drug discovery and drug development program. Recent emphasis has been placed on increasing sample throughput while, at the same time, increasing information content within assays. To this end, screening for potential drug-drug interactions, overall metabolic stability, and metabolite profiles is used early in discovery to select compounds for development.

The throttle on the metabolism discovery engine is limited by the time required for data processing and reporting of the information-rich assays used in discovery-stage metabolism studies. To address this need for increased throughput screening in drug discovery, novel liquid chromatography (LC) and mass spectrometry (MS) platforms, with informatics that are suited to the specific task, comprise the necessary analytical toolset to maximize output.

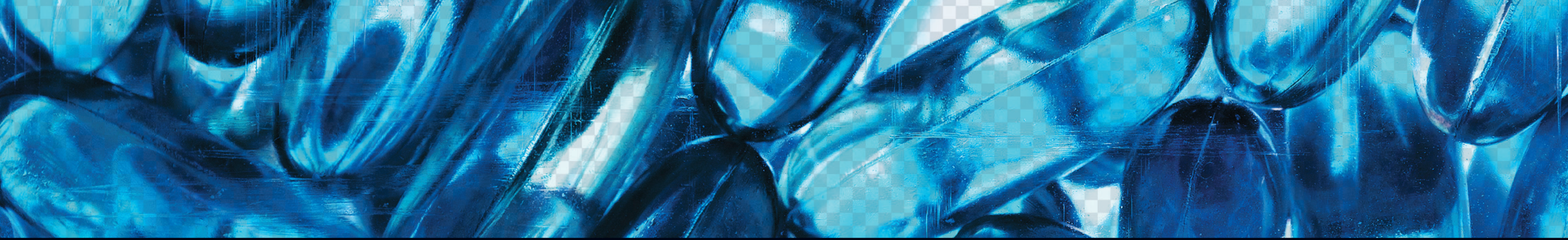
The pharmaceutical industry is undergoing more changes than ever before, driven by aggressive competitive and technological landscapes. Ever-decreasing cycle times and cost-cutting provide the impetus for innovative R&D partnerships and outsourcing that are reshaping the business strategies of many pharmaceutical and biotechnology companies. Finding new ways to increase productivity, decrease costs, and develop new therapies that will enhance both human health and shareholder satisfaction are mandates.

Central to this challenge are questions about which strategies are most effective for drug discovery laboratories to reduce the time required to develop and market new drugs. Studies of drug metabolism play a vital role in the pharmaceutical industry. LC/MS is the standard used across all areas of drug discovery to provide early data that might help scientists understand the metabolic fate or liabilities of a drug candidate, and thus, save time and reduce costs in the long-term.

The identification of *in vitro* and *in vivo* drug metabolites is, indeed, part of the discovery and development programs of all pharmaceutical and biotechnology companies. During early absorption, distribution, metabolism, and excretion (ADME) studies, scientists develop and utilize *in vitro* models and rapid methods to evaluate (bio)pharmaceutical properties and ADME parameters to support lead optimization and drug formulation development.

In this realm, the ultimate goal is to develop faster, more accurate, and more relevant high-throughput models that correlate *in vitro* parameters with *in vivo* pharmacokinetics (*in vitro*–*in vivo* correlation). To accomplish this, an in-depth, mechanistic understanding of the processes involved is required.

Waters technologies are ever-evolving in response to the increasing analytical demands of metabolism studies. Chromatographic speed derived from the ACQUITY UPLC® System addresses the demands of high-throughput screening in drug discovery. SYNAPT™ Mass Spectrometers improve levels of detection to deliver consistently better quality results, such as increasing the accuracy of mass measurement to confirm the presence of a putative metabolite.



These LC/MS innovations are accompanied by excitement over the possibilities of how they can either improve laboratory workflow or provide more informative data. However, recognizing that more data often results in a backlog in data processing and reporting, Waters solutions are developed to not only address analytical quality and throughput, but also to facilitate the interpretation of these complex datasets – to extract information that is most relevant for meaningful decision-making. Waters LC/MS system solutions for metabolism studies are proving most powerful when they are strategically combined with informatics that help researchers get to concise and contextual data.

Automated software algorithms available with MetaboLynx™ XS Software detect biotransformations for expected and unexpected metabolites. Samples from either *in vitro* incubations or *in vivo* dosing experiments can now be analyzed quickly by UPLC®/MS, followed by a multi-dimensional data search that correlates retention time, mass-to-charge ratio (m/z value), intensity, and components from alternative detection technologies (e.g., diode array UV and radiochemical monitoring). The combination of chemically intelligent tools, such as dealkylation software that helps to drive the automatic generation of mass defect filters, aids in the interpretation of results. The Mass Defect Filter assists with the comparison of data from the analyte with the control sample for rapid filtering of matrix-related peaks, which would otherwise produce an unmanageable list of false metabolite peaks. Accurate mass further aids removing false positives, allowing the scientist to eliminate non-drug-related peaks more quickly and with greater confidence. Then MassFragment™ automates metabolite fragment structure assignment and confirmation to complete the structural elucidation process (Figure 1).

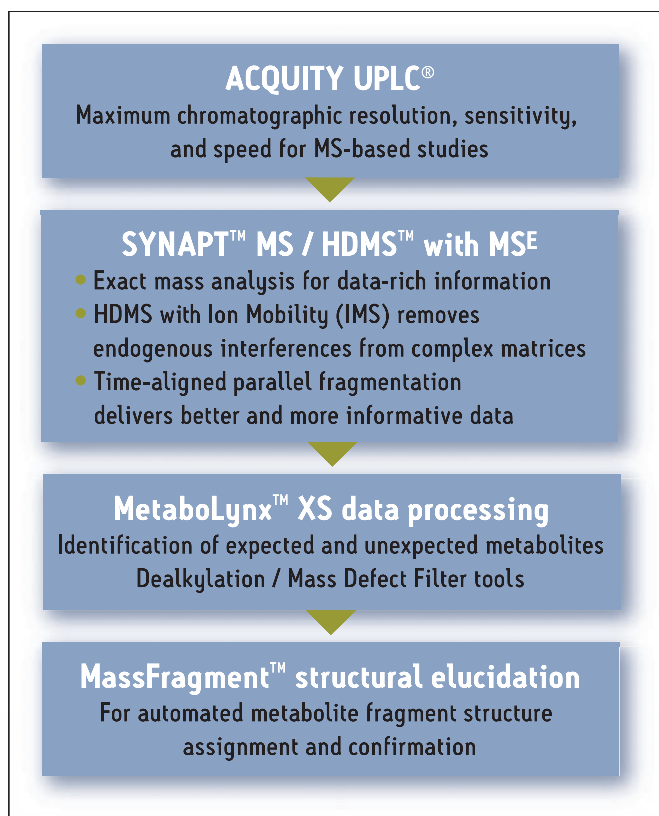
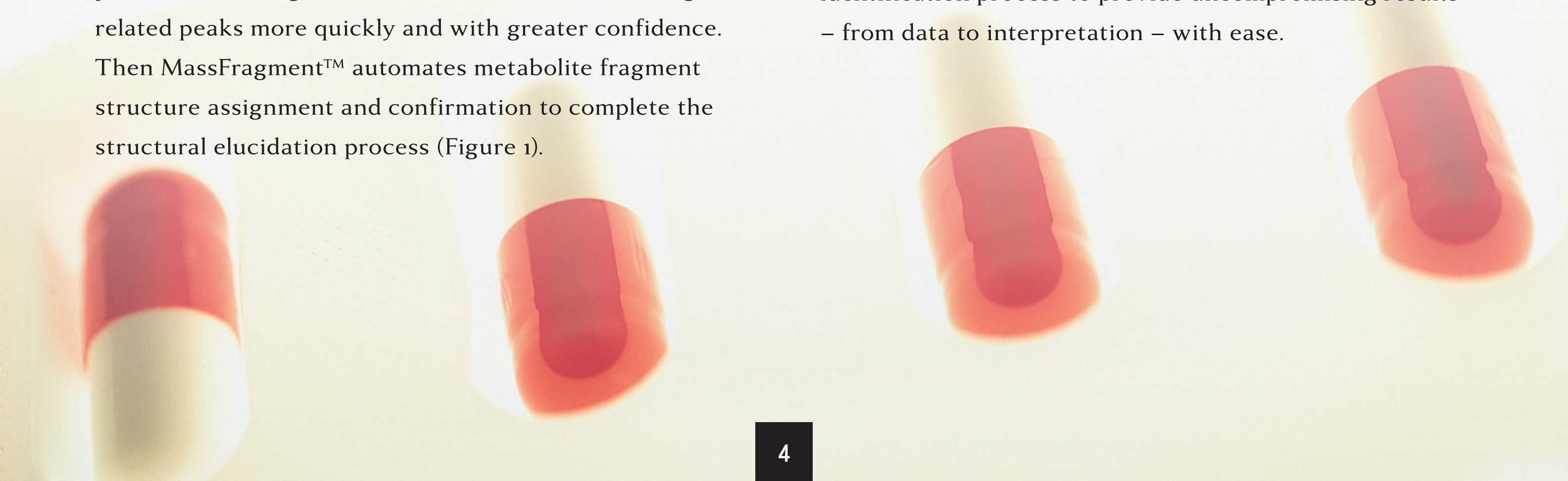


Figure 1. Process for metabolite identification.

There are a plethora of solutions on the market that claim to address the specific workflow needs of the drug metabolism scientist. In reality, the majority of these deliver disconnected pieces of information with little regard for the end scientific goal. When facing mounting pressures to produce more and more within compressed timeframes, Waters solutions uniquely address the entire metabolite identification process to provide uncompromising results – from data to interpretation – with ease.



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AN ULTRA RAPID AND SENSITIVE STRATEGY FOR *IN VIVO* METABOLITE IDENTIFICATION FOR PROTOPANAXADIOL FROM RAT BILE: UPLC VS. HPLC

Jose Castro-Perez, Kate Yu, John Shockcor, Henry Shion, Yanguo Sun, and Emma Marsden-Edwards
Waters Corporation, Milford, MA, U.S., Shanghai, China, and Manchester, UK

Yuya Wang, Xiaoyan Chen, Dafang Zhong
Shanghai Institute of Materia Medica, Chinese Academy of Science, Shanghai, China

INTRODUCTION

In vivo drug metabolite identification studies encompass some of the most difficult analytical challenges, such as extremely complicated biological matrices and a lack of pure standards for putative metabolites. Utilization of a high resolution chromatographic system plays an important role in separating metabolites from endogenous matrices.

The advent of Waters UltraPerformance LC® (UPLC®) technology has enabled chromatographic separations to be routinely performed on columns packed with sub-2 µm particles at high linear velocity. Such separations result in high resolution, excellent throughput, and high sensitivity chromatographic analyses.[1-2] These analytical qualities ensure the separation of coeluting metabolites and minimize ESI matrix effects. The use of oa-TOF mass spectrometry offers fast acquisition rates that are compatible with UPLC, high resolution, and exact mass measurement for confident compound identification.

Protopanaxadiol is one of the major sapogenin for ginsenosides[3] (Figure 1). It has been reported that this compound may inhibit cancer cell growth.[4] However, its metabolism in animal or human has not yet been reported. A previously-described UPLC/TOF-MS^E workflow including MetaboLynx™ and MassFragment™ software for metabolite identification[1] was applied to this study and adapted so that an equivalent workflow using a HPLC separation could be compared.

This application note describes a study that demonstrates the advantages of UPLC over traditional HPLC using TOF-MS detection. An *in vivo* drug metabolism experiment was performed using protopanaxadiol (PPD). A rat was dosed with PPD, with bile subsequently collected and analyzed by both UPLC/TOF-MS and HPLC/TOF-MS.

We show that the higher resolution and sensitivity of UPLC results in the identification of more metabolites, with significantly enhanced signal-to-noise ratios in a shorter timeframe than can be achieved using a comparable workflow using HPLC.

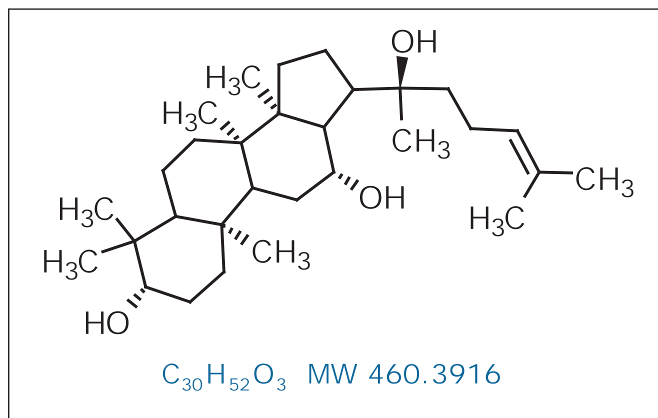

Figure 1. Chemical structure of the protopanaxadiol (PPD).

METHODS

Dosing the animal

Male Sprague-Dawley rats were fasted for a 12-hour period prior to dosing. Blank bile was collected prior to dosing. The PPD oral dose amount was 100 mg/kg. The dose bile was collected three to six hours after administration of a single dosage.

Sample preparation

400 µL of MeOH was added into a 200 µL aliquot of rat bile. After being vortex-mixed and centrifuged, the supernatant was evaporated to dryness and reconstituted later with 1 mL of ACN/H_2O (2:8), centrifuged again at 13,000 RPM, and the supernatant was injected.

MS conditions

MS system:	Waters SYNAPT™ HDMS™ System
Scan range:	100 to 1000 Da
Source temp.:	120 °C
Desolvation temp.:	420 °C
Cone voltage:	15 V
Collision energy (CE):	Low CE: Trap 2 eV/Transfer 0.5 eV High CE: Trap 10 to 25 eV/Transfer 4 eV

HPLC conditions

Instrument:	Waters ACQUITY UPLC® System
Column:	Zorbax Extend-C_{18}, 4.6 x 150 mm, 5 µm
Column temp.:	45 °C
Sample temp.:	4 °C
Mobile phase A:	5 mM Ammonium acetate buffer
Mobile phase B:	Acetonitrile
Flow rate:	0.8 mL/minute
Injection vol.:	10 µL

Gradient:

Time (min)	%A	Curve
0	90	
5.5	80	6
10	80	6
10.5	60	6
15	60	6
15.5	40	6
20.0	40	6
20.5	20	6
60	20	6
61	90	1
71	90	1

UPLC conditions

Instrument:	Waters ACQUITY UPLC® System
Column:	ACQUITY UPLC HSS T3, 2.1 x 100 mm, 1.7 µm
Column temp.:	45 °C
Sample temp.:	4 °C
Mobile phase A:	5 mM Ammonium acetate buffer
Mobile phase B:	Acetonitrile
Flow rate:	0.6 mL/minute
Injection vol.:	10 µL

Gradient:

Time (min)	%A	Curve
0	90	
11	30	6
13	10	1
15	90	1

Data processing: MetaboLynx XS Application Manager with MassFragment

RESULTS

The analysis of protopanaxadiol in bile gave rise to an important number of Phase I and II metabolites. The major biotransformation detected corresponded to the O-sulfate conjugated metabolite. The amount of all O-sulfate conjugates equated to 51% of the total metabolites identified in this study.

Further investigation of the results revealed why UPLC has a noticeable advantage over HPLC for metabolite identification. Figure 2 shows two extracted ion chromatograms (XIC) for the O-sulfate metabolites (m/z 557.351) with an extraction window set at ± 30 mDa.

- Nine O-sulfate metabolites were detected by UPLC (Figure 2A).
- Five O-sulfate metabolites were detected by HPLC (Figure 2B).

In contrast to the HPLC analysis, Figure 2 highlights the power of the UPLC strategy and its additional benefits – the UPLC analysis not only resulted in more identified metabolites, but it also provided a 5.3-fold improvement in signal-to-noise and 1.6-fold improvement in chromatographic resolution.

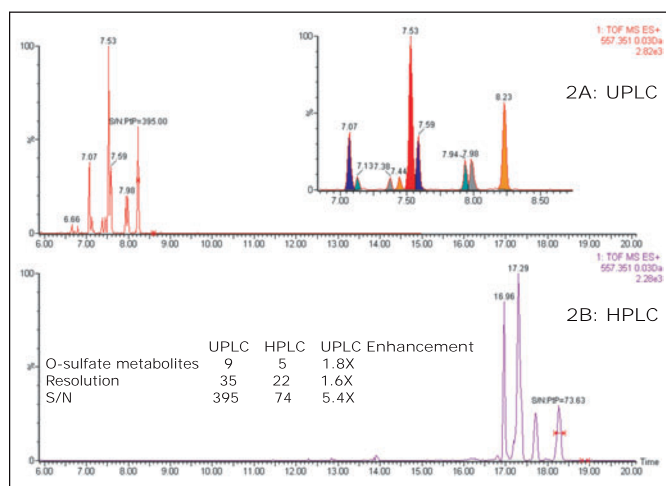

Figure 2. XICs of O-sulfate metabolites for protopanaxadiol, including 2A, the XIC obtained from the UPLC run, and 2B, the XIC obtained from the HPLC run.

Table 1 summarizes the comparison of the metabolite identification results between the UPLC and the HPLC methods. From the data shown, it is apparent that with the UPLC approach, simultaneous enhancements in speed, resolution, and sensitivity were observed. As a result, the total number of metabolites identified was significantly higher when utilizing the UPLC separation in both ionization modes (positive and negative ESI).

The enrichment factor for UPLC vs. HPLC in positive ion mode was +56%, and in negative ion mode, +59%. This may be attributed to the fact that more metabolites and endogenous components were resolved chromatographically, giving rise to an increased degree of separation and a reduction in ion suppression.

PPD IN RAT BILE	UPLC	HPLC	ENRICHMENT FACTOR BY UPLC
Metabolites ESI+	39	25	56%
Metabolites ESI-	35	22	59%
LC run time (min)	15	71	4.7X faster

Table 1. Comparison of metabolite identification results obtained from UPLC and HPLC.

Figure 3A shows a chromatogram that combines the extracted masses for all of the metabolites identified using UPLC/MS. The time period between 4 to 12.3 minutes is shown as this is where all of the metabolites were eluted. Figure 3C shows a similar chromatogram for the HPLC/MS data. Note the time scale shown is 11 to 21.5 minutes.

UPLC offers a much better peak fidelity and resolution in a more compact time frame. Figure 3B shows the comparison of the UPLC and the HPLC combined metabolite chromatograms on the same time scale. The total separation time for UPLC was 15 minutes, while the total separation time for HPLC was 71 minutes. The UPLC analysis time was almost five times shorter, and resulted in about a 1.6-fold increase in identified metabolites than the HPLC methodology.

Thus, UPLC enables enhanced productivity with better quality data.

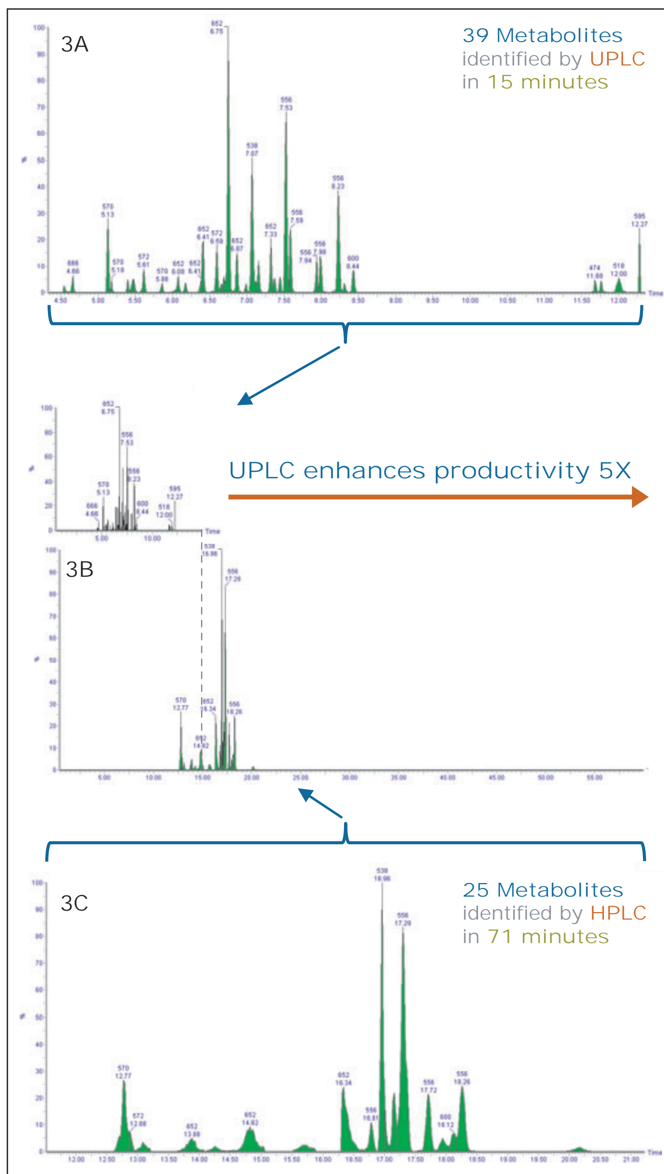

Figure 3. Chromatographic comparison of the UPLC metabolite ID results vs. the HPLC metabolite ID results.

CONCLUSION

This application note demonstrates a simple and effective workflow combining UPLC/TOF-MS^E with MetaboLynx and MassFragment software for Traditional Herbal (Chinese) Medicine metabolite identification from rat bile. Compared with HPLC, the UPLC approach delivered much higher chromatographic resolution (1.6X increase) in a shorter time frame. In turn, this directly translates to more productivity with more samples to be analyzed per unit time (5X increase), and with more metabolites being identified (1.6X increase).

The enrichment factor obtained from UPLC over HPLC for the detection of metabolites was the direct result of enhanced chromatographic resolution. This subsequently means that it is possible to obtain better and more conclusive MS/MS data from isobaric metabolites that would have otherwise coeluted. Therefore, this comprehensive workflow for metabolite identification enables the scientist to be more adept in the structural elucidation step.

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Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com

MAINTAINING HIGH SPECTRUM RESOLUTION WITH INCREASING SCAN SPEEDS FOR METABOLITE IDENTIFICATION USING UPLC WITH THE SYNAPT MS

Kate Yu, Jose Castro-Perez, Robert S. Plumb
Waters Corporation, Milford, MA, U.S.

INTRODUCTION

The detection and identification of candidate pharmaceuticals and their metabolites in biological fluids is an essential part of the drug discovery and development process. UltraPerformance LC® (UPLC®) coupled with mass spectrometry (MS) sets a new standard in the discipline of metabolite identification, producing superior chromatographic separations, high throughput, and increased sensitivity.

The performance of Waters® SYNAPT™ MS Mass Spectrometer is ideal for the challenging requirements of a metabolite identification study. It offers high spectral resolution with exact mass measurement for both MS and MS/MS analyses. As a result, positive confirmation of analyte identity is obtained in complex matrices from a single injection.

Reliable accurate mass measurement is critically dependent on the spectral resolution, which brings operational parameters into consideration when coupled with the ACQUITY UPLC® System. Typical peak widths generated from UPLC separations are in the range of 1-3 seconds at the base. High acquisition speeds are required to ensure good peak definition. However, fast data acquisition often results in reduced spectral quality and reduced mass accuracy with many mass spectrometers.

This application note shows the consistent high spectral resolution obtained from the SYNAPT MS with increasing data acquisition speeds. This is demonstrated using *in vitro* microsomal incubations of buspirone. All analyses were performed with the ACQUITY UPLC and SYNAPT MS.

Figure 1. The ACQUITY UPLC System with the SYNAPT MS for metabolite identification.

EXPERIMENTAL

In vitro microsome incubation

The parent drug buspirone was incubated with human and rat liver microsomes at a 100 µM level. The incubation was carried out at 37 °C, in a solution of 50 mM potassium phosphate adjusted to pH 7.4 containing the appropriate co-factors. The reaction was terminated after 90 minutes with two volumes of cold acetonitrile to one volume of sample. The samples were stored frozen at -20 °C and diluted with water in a one-to-one ratio prior to UPLC/MS analysis.

UPLC conditions

LC system:	Waters ACQUITY UPLC System
Column:	ACQUITY UPLC BEH C_{18}, 1.7 µm 2.1 mm I.D. x 100 mm
Column temp.:	65 °C
Mobile phase:	A: Water + 0.1% formic acid B: Acetonitrile + 0.1% formic acid

Gradient:

Time (min)	Flow (mL/min)	%A	Curve
0.00	0.600	98.0	
3.50	0.600	30.0	6
4.00	0.600	0.0	1
7.00	0.600	98.0	1

MS conditions

MS system:	Waters SYNAPT MS Mass Spectrometer
Ionization mode:	ESI+
Cone voltage:	30 V
Capillary voltage:	3 kV
MS mode:	MS full scan in W-mode
Desolvation temp.:	450 °C
Source temp.:	120 °C
Collision energy:	5 eV

RESULTS AND DISCUSSION

Buspirone (MW 385) is an anti-anxiety drug with hydroxylation as one of its major metabolic pathways[1]. Figure 2 shows the extracted ion chromatograms for the M+16 buspirone metabolites at m/z 402.

In order to study the effect of fast data acquisition speeds on spectral resolution, the same sample was injected multiple times by employing the same UPLC/MS protocol. The data acquisition speed was varied from injection to injection. Figure 2 clearly shows that higher scan speed data capture experiments gave much better peak definitions. Data points are important for analytical precision, especially if quantitative or semi-quantitative results are required for the analysis.

Figure 3 shows the mass spectra obtained for the m/z 402 ion eluting at 1.6 minutes. The spectral resolution was maintained at approximately 20,000 FWHM (full width at half maximum) regardless of the data acquisition speed. The consistent high resolution obtained by SYNAPT MS is essential for high quality exact mass measurement during sample analysis, making confirmation of peak identity a much easier task.

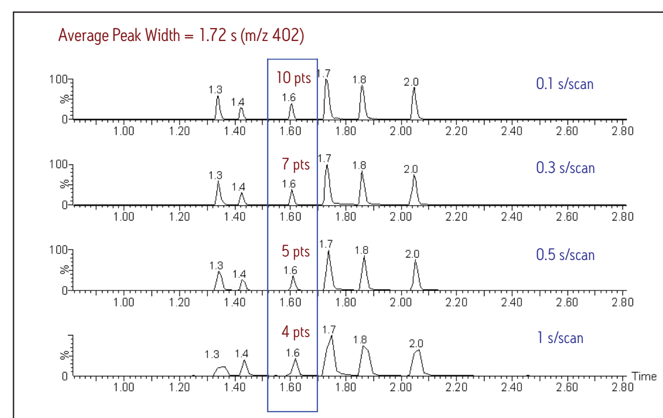

Figure 2. Selected ion chromatograms for the M+16 buspirone metabolites at m/z 402 obtained at different scanning speeds. More data points were collected as the scan speed increased resulting in better peak definition.

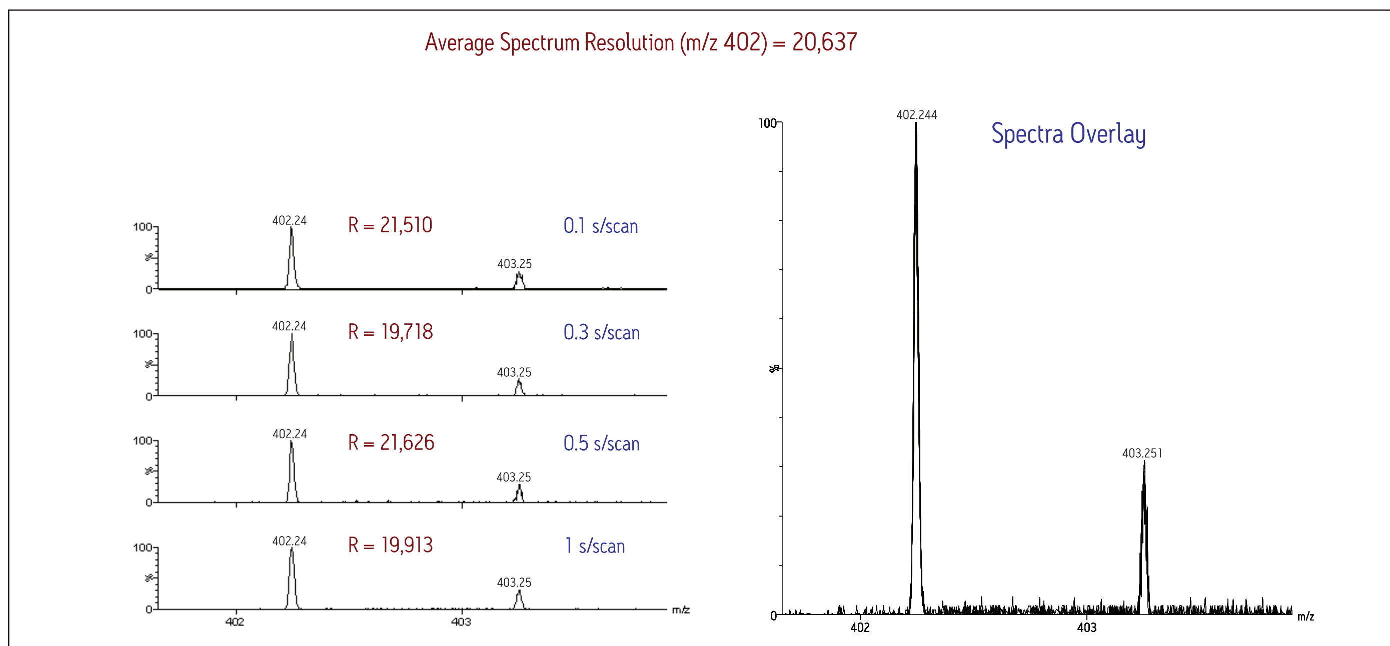

Figure 3. The mass spectra for buspirone metabolites (M+16) showing spectral resolution obtained at different acquisition speeds.

CONCLUSION

We have demonstrated that the SYNAPT MS has superior and consistent spectral resolution with 20,000 FWHM at scan speeds ranging from 1 to 0.1 scans/second. This makes the SYNAPT MS an ideal mass spectrometer for structural analysis. When coupled with the ACQUITY UPLC, metabolite identification can be performed rapidly with no loss in data quality.

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34 Maple Street
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T: 1 508 478 2000
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OBTAINING MAXIMUM INFORMATION WITH A NOVEL WORKFLOW FOR *IN VIVO* METABOLITE DETECTION AND IDENTIFICATION USING SYNAPT HDMS

Jose Castro-Perez, Kate Yu, and John Shockcor
Waters Corporation, Milford, MA, U.S.

INTRODUCTION

In vivo sample analysis for metabolite identification is extremely challenging due to the lack of radio labeled drugs in early discovery and the high level of endogenous biological non-drug-related interferences. As a result, there are no reference points for identifying xenobiotics *a priori*. Analysts rely heavily on personal experience and analytical strategies to detect and identify low-level metabolites.

In principle, some of these problems may be reduced by utilizing an additional stage of separation. This separation stage is orthogonal to LC and mass spectrometric separations and occurs on an intermediate timescale between the two separations. The Waters® SYNAPT™ HDMS™ System provides this capability by combining high-efficiency ion mobility-based measurements and separations with tandem mass spectrometry.[1]

HDMS mode on the SYNAPT HDMS System employs ion mobility-based separations (IMS) that separate ionic species as they drift through a gas under the influence of an electric field. The rate of the drift depends on the following factors: the mass of the ion, its charge state, and the interaction cross-section of the ion with the gas. Consequently, it is possible to separate ions with the same nominal m/z value if they have different charge states or sufficiently different interaction cross-sections.

In this study, we investigate use of the SYNAPT HDMS System for *in vivo* drug metabolite analysis. SYNAPT HDMS allows the user to operate the system in either time-of-flight (TOF) mode or HDMS mode. Apart from the orthogonal separation afforded by high-efficiency ion mobility, this configuration also has the ability to fragment ions in the Triwave™ region: pre-IMS, post-IMS, or both combined in parallel as shown in Figure 1.[2]

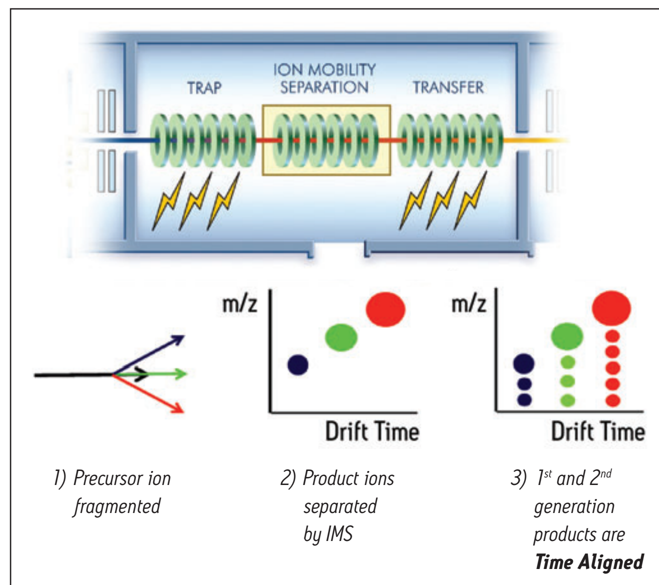


Figure 1. Illustration of the time-aligned parallel fragmentation (CID-IMS-CID) described in this experiment.

The combination of pre- and post-IMS fragmentation, also referred to as time-aligned parallel (TAP) fragmentation, provides a highly informative fragmentation spectra that contains first- and second-generation fragment ions with none of the low-mass cutoffs that are observed in conventional ion traps.

For a given TAP experiment, ions of interest are selected in the quadrupole region. The ions are then fragmented in the Trap region using CID energy. These first-generation fragment ions are next separated in the ion mobility T-Wave.™ Each first-generation fragment ion has a different drift time depending on the factors described above.

As these fragmented ions emerge from the ion mobility region, they are subjected to a further stage of fragmentation in the T-Wave Transfer region before entering the TOF region, generating second-generation fragment ions. The drift time generated by each of the first-generation fragment ions is used to localize and align which fragment was responsible for producing the second-generation fragment ions.

To facilitate the analysis of this data, Waters MassFragment™ Software, a structure elucidation tool, can be used to rationalize fragment ions quickly. This software enhances the entire HDMS analytical workflow (Figure 2) by reducing structure elucidation time, which is one of the major bottlenecks of *in vivo* metabolite identification.

With this workflow-based approach to metabolite analysis using HDMS, a straightforward two-injection strategy is necessary for fraction collection, resulting in more valuable time spent analyzing and optimizing the sample analysis conditions for each fraction.

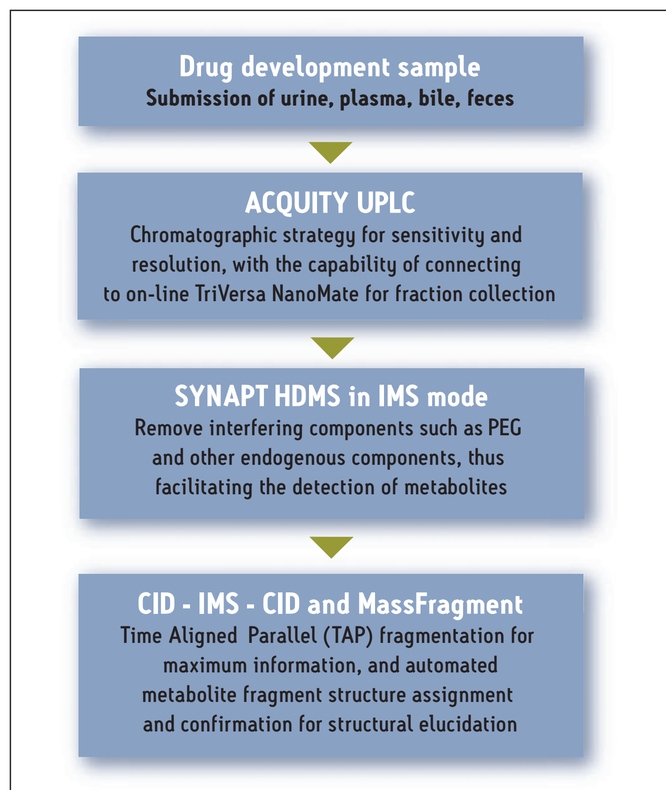


Figure 2. The end-to-end workflow approach to in vivo *metabolite identification sample analysis.*

EXPERIMENTAL

Sample preparation

At a time point of 4 hours, rat urine sample was collected from a 5 mg/kg (verapamil) oral dose experiment. The sample was diluted 1/4 with water + 0.1 % formic acid and injected directed to the LC/MS.

LC conditions

LC system:	Waters ACQUITY UPLC® System
Column:	Waters ACQUITY UPLC HSS T3 Column 2.1 x 100 mm, 1.7 µm
Column temp.:	45 °C
Flow Rate:	600 µL/min
Mobile Phase A:	Water + 0.1% formic acid
Mobile Phase B:	Acetonitrile
Gradient:	0 to 50% B linear in 10 min, 50% B to 10% B linear in 1 min, hold at 10% B for 1 min, Re-equilibrate at 0% B for 3 min
Run time:	15 minutes

Fraction collection

System:	Advion TriVersa NanoMate
UPLC flow:	600 µL/min
Flow split:	2000:1
Collection plate:	96-well plate
Collection time:	7 s per well (70 µL collected per well)
Trigger:	Fraction collector was triggered by time

MS conditions

MS system:	Waters SYNAPT HDMS System
Ionization mode:	ESI positive
Capillary voltage:	3200 V
Cone voltage:	35 V
Desolvation temp:	400 °C
Desolvation gas:	800 L/Hr
Source temp:	120 °C
Acquisition range:	50 to 1000 m/z
HDMS gas:	Helium
Collision gas:	Argon

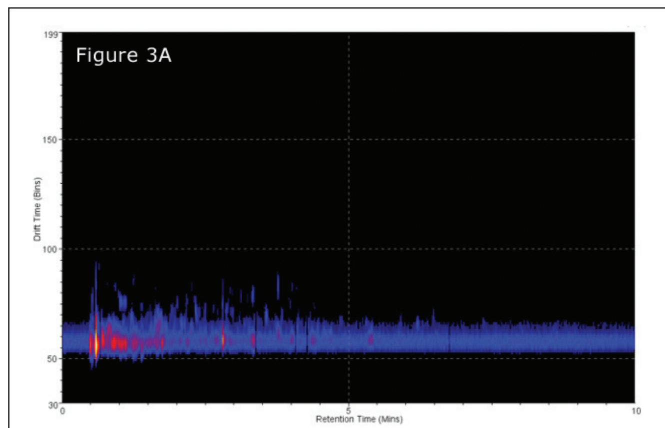
RESULTS AND DISCUSSION

By way of sample comparison, the differences between the control (Figure 3A) and analyte (Figure 3B) can be easily visualized as highlighted in white circles. The DriftScope™ Software tool utilized for data interrogation allowed lassoing of the drift time regions of interest. Thus it was possible to obtain a clean extracted ion TIC only corresponding to the metabolites of interest.

The ability to select only the metabolites of interest in the extracted TIC is shown in Figure 4. Since the metabolites are above the chemical noise and background ions, the resulting TIC was very clean with zero baseline noise. This made detecting putative metabolites much easier.

Once the metabolites of interest were found, the fractions for the peaks of interests were collected by the use of the TriVersa NanoMate workstation (Advion, Ithaca, NY, U.S.). For each of the fractions collected, a time-aligned parallel (TAP) fragment experiment was carried out.

After carrying out TAP fragmentation on the parent drug (Figure 5), every drift time region was interrogated independently by creating fragmentation drift time trees. It was not necessary to pre-select precursor ions for the CID-IMS-CID experiments as all ions emerging from the ion mobility cell were fragmented in parallel.



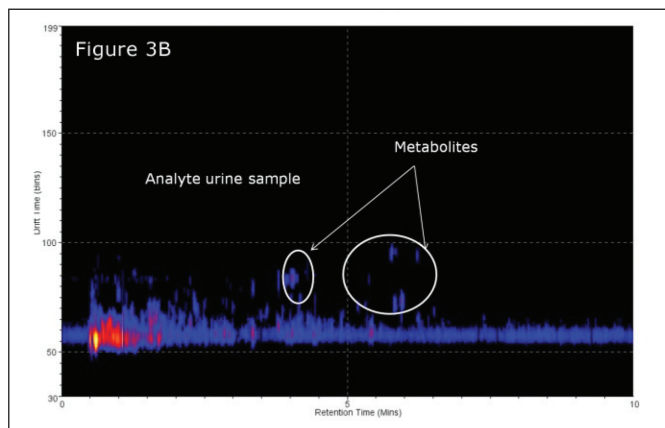


Figure 3. Drift time plot for control sample (3A) and for analyte sample (3B) showing drift time (x-axis) vs. retention time (y-axis).

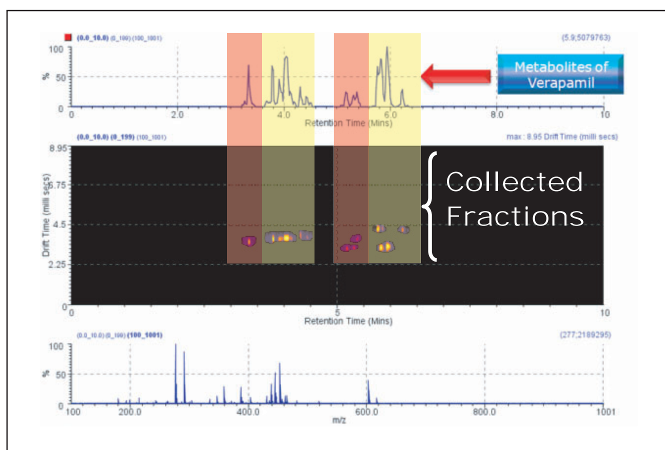


Figure 4. Excised corresponding potential metabolite drift times from comparison of control and analyte sample drift plots.

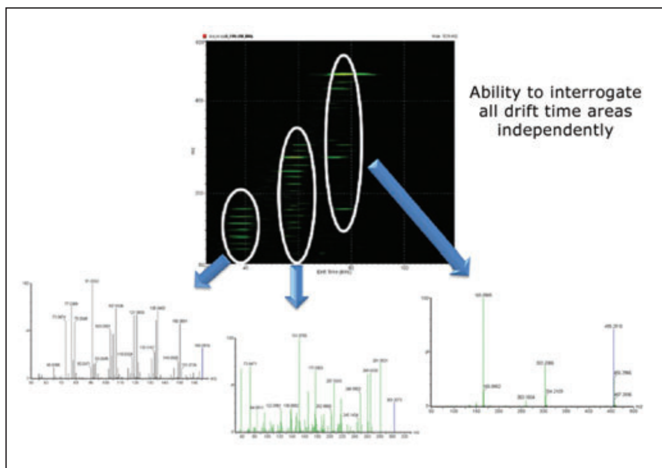


Figure 5. TAP fragmentation (CID-IMS-CID) for the verapamil parent drug. Each of the drift time areas can be interrogated separately.

The major fragment ions obtained were then submitted to MassFragment Software. This software tool enabled us to elucidate the structure of the parent compound, as shown in Figure 6.

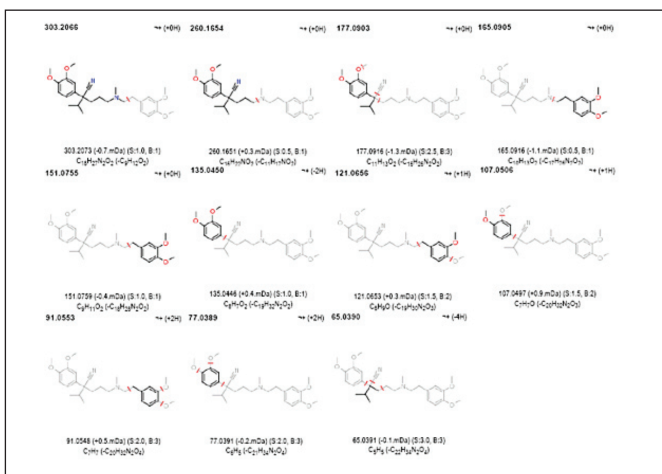


Figure 6. Interpretation of results with MassFragment from TAP fragmentation (CID-IMS-CID) for the verapamil parent drug.

Once the parent fragment ions were characterized, it was possible to localize the sites of chemical modification for some metabolites. For instance, the site of one of the O-desmethyl glucuronidated metabolites for verapamil was identified by the use of TAP fragmentation (Figure 7).

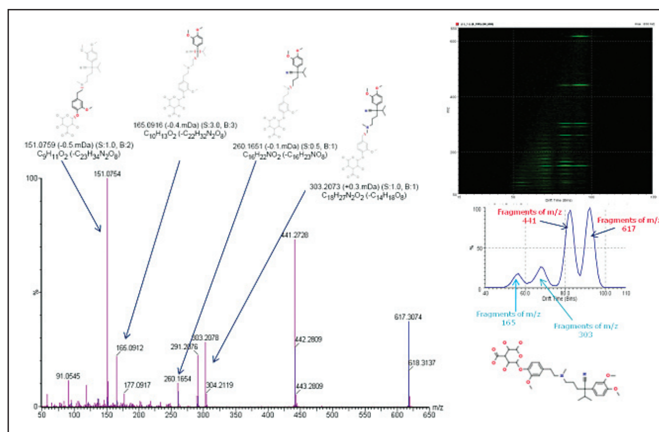


Figure 7. TAP fragmentation (CID-IMS-CID) for the O-desmethyl glucuronidated metabolite of Verapamil.

CONCLUSION

- Utilizing the SYNAPT HMDS System in HDMS mode allows data obtained from complex *in vivo* matrices to be dissected with greater specificity by utilizing an additional dimension (drift time) of information. This makes it possible to remove chemical noise and other interferences, such as PEG, thus facilitating the search for putative metabolites.
- The configuration of the TriVersa NanoMate allows TAP fragmentation experiments to be carried out in a unique but informative way as all ions are fragmented in a parallel fashion.
- The use of the chemically-intelligent software tool MassFragment, for structure elucidation, is very effective in reducing the data reviewing bottleneck as it allows rapid compound identification.
- Overall, the powerful features of the SYNAPT HDMS System enable scientists to improve both their productivity and the amount of key information necessary to make quick decisions in a timely manner.

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1. Pringle SD, Giles K, Wildgoose JL, Williams JP, Slade SE, Thalassinos K, Bateman RH, Bowers MT, Scrivens JH. An Investigation of the Mobility Separation of Some Peptide and Protein Ions using a New Hybrid Quadrupole Traveling Wave IMS oa-TOF Instrument. International Journal of Mass Spectrometry. March 2007; 261 (1): 1-12.

2. The traveling wave device described here is silimar to that described by Kirchner in U.S. Patent 5,206,506 (1993).

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REMOVAL OF INTERFERENCES AND EASIER METABOLITE DETECTION BY ION MOBILITY MASS SPECTROMETRY

Jose Castro-Perez, Kate Yu, and John Shockcor
Waters Corporation, Milford, MA, U.S.

INTRODUCTION

The task of identifying drug metabolites from complex biological matrices such as bile, plasma, feces and urine with traditional techniques can be difficult. One of the typical problems when running *in vivo* samples is that without the use of radiolabeled compounds, there are no reference points to look for xenobiotics. In the majority of cases, the analyst relies heavily upon personal experience and customized analytical strategies to detect and identify low-level metabolites from high endogenous backgrounds.

The complexity of this analysis could be significantly reduced by the use of an additional stage of separation that is orthogonal to the LC and mass spectrometric separations, and occurs on a timescale that is intermediate between the two. A technique that possesses this capability is ion mobility spectrometry (IMS).

IMS separates ionic species as they drift through a gas under the influence of an electric field. For any particular ion, the rate of drift depends on its mobility, which in turn is dependent on factors such as mass, charge state and the interaction cross-section of the ion with the gas. This additional dimension of separation fidelity leads to improved specificity and sample definition so that more information about the sample can be extracted. The multi-dimensional data produced by the Waters® SYNAPT™ High Definition MS™ (HDMS™) System is visualized and manipulated using DriftScope™ Mobility Environment Software.

In this work, UPLC®/IMS-TOF-MS analysis was conducted on a rat bile sample. By using DriftScope Software, the metabolites from this complex matrix were easily visualized, as the drift time was used to separate background ions from real drug-related metabolites. Further extracted ion chromatograms were also obtained by selecting specific ions with the software. As a result, the extracted ion chromatogram and MS spectrum for each metabolite were attained without interference from the endogenous compounds.

Figure 1. The ACQUITY UPLC System with the SYNAPT High Definition Mass Spectrometry System.

EXPERIMENTAL

The *in vivo* ketotifen sample was obtained from rat bile. The drug was dosed intraperitonally at 10 mg/kg and bile was collected from 0 to 3 hours after drug administration. The sample was diluted 1/10 using water with 0.1 % formic acid prior to the UPLC/MS analysis.

LC conditions

LC system:	Waters ACQUITY UPLC® System
Column:	ACQUITY UPLC BEH C_8 Column 2.1 x 100 mm, 1.7 µm
Column temp.:	45 °C
Flow rate:	600 µL/min
Mobile phase A:	Water + 0.1% formic acid
Mobile phase B:	Acetonitrile
Gradient:	95% A to 20% A in 6.5 min, hold at 0% A for 0.5 min before returning to 95% A for re-equilibration

MS conditions

MS system:	Waters SYNAPT HDMS System
Ionization mode:	ESI Positive
Capillary voltage:	3200 V
Cone voltage:	35 V
Desolvation temp.:	400 °C
Desolvation gas:	800 L/hr
Source temp.:	120 °C
Acquisition range:	100 to 1000 m/z
Mobility carrier gas:	Nitrogen at 32 mL/min

RESULTS

The UPLC/IMS-TOF-MS results were reviewed using the DriftScope Software. It allowed sample data to be visualized as mz/dt, or dt/rt, or mz/rt (mz=m/z, dt=drift time, and rt=retention time). The initial review using the dt/rt option enabled the visualization of all components by using drift time, which was used to exclude false positives and consequently remove interfering ions that masked the detection of smaller metabolites.

As shown in Figure 2, this was a very complex sample. But due to the fact that the drift time information was available, this data dimension was used to select the areas of interest. This can be seen in area A of the figure, where all of the metabolites and the parent drug reside. A simple lasso around this region within the software allowed efficient export of the corresponding total ion chromatogram (TIC) to MassLynx™ Software for further data processing.

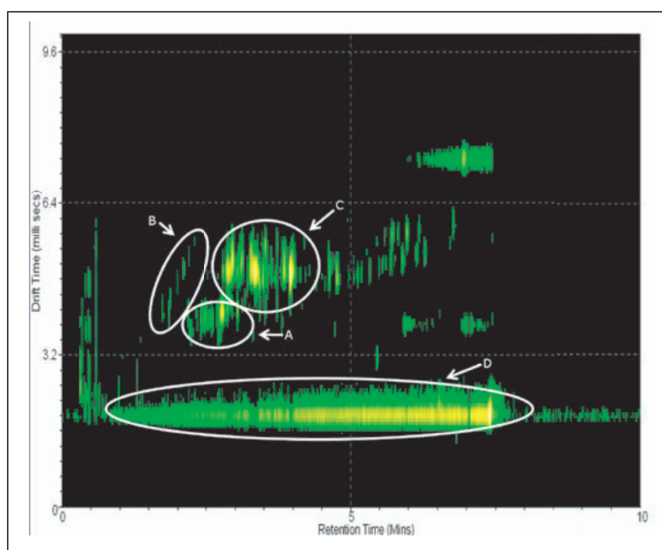

Figure 2. DriftScope Software's dt (msec)/rt (min) plots for rat bile dosed with ketotifen. Various regions are easily visualized from the plots: (A) metabolites and the parent drug, (B) PEG, (C) bile salts, and (D) solvent ions.

Also in Figure 2, when focusing on areas A and B, it was observed that even though some of the metabolites co-eluted chromatographically with bile salts, they both exhibited different drift times. This indicated a very selective technique to remove the background ions (D), PEG (B), and bile salt interference (C). Figure 3 shows that once the area of interest had been selected and exported to MassLynx, substantial differences were seen in the cleanup of background and bile salt interferences when drift time was used to extract the metabolite information (B) vs. a normal TIC without the use of IMS (A).

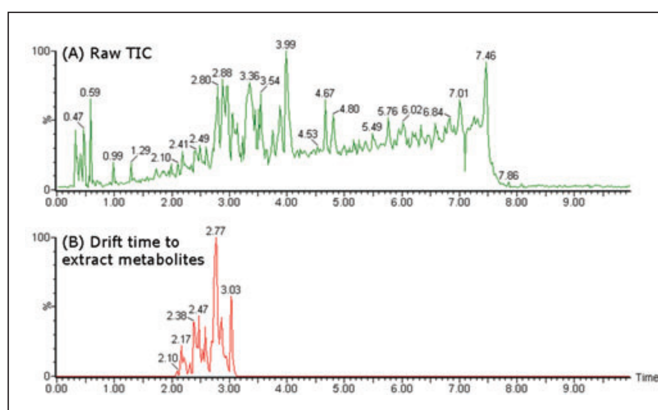

Figure 3. TICs for ketotifen rat bile sample: (A) TIC without the use of drift time to extract the information, and (B) TIC with the use of drift time to extract the information.

Another interesting observation in Figure 2 was that as only area A was selected, we were consequently above the background noise which was constituted mainly by solvent ions (D) so the noise was zero. Therefore, it becomes even easier to detect very small components in complex samples where the background is typically very high.

Further, we combined the TICs from the non-IMS acquisition vs. those from the IMS-based acquisition from 2 to 3.2 minutes where both the metabolites and the parent drug eluted. As seen in the results displayed in Figure 4, it is clear how much more visible the metabolites were, showing an overall improvement in the corresponding spectra when drift time was employed. Even though some of the bile salts had the same retention times, drift time very effectively removed them from the spectra to further aid in the detection of metabolites.

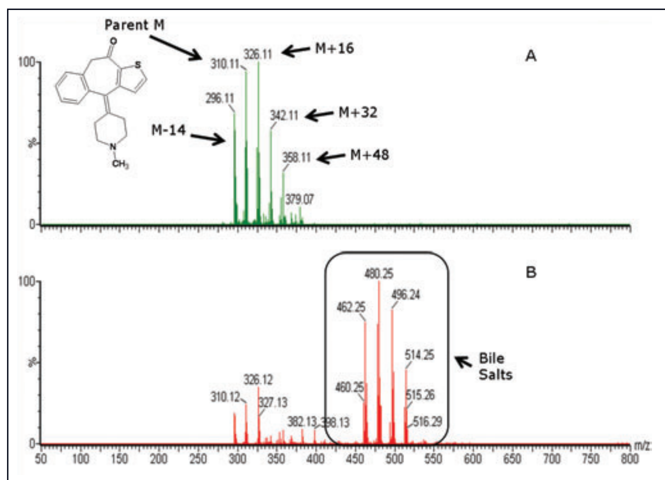

Figure 4. Spectra combined over retention times from 2 to 3.2 min. for both TICs: (A) with the use of drift time to extract the information, and (B) without the use of drift time to extract the information.

Therefore, from Figure 4 (A) it was deduced that ketotifen at m/z 310 was detected together with all of its major metabolites in bile corresponding to N-dealkylation m/z 296 (-CH2), hydroxylation m/z 326 (+O), double hydroxylation m/z 342 (+2O) and triple hydroxylation m/z 358 (+3O).

Other minor metabolites were also detected, indicating hydroxylation and reduction to ketone m/z 324 (+O -2H), and double hydroxylation and reduction to ketone m/z 340 (+2O - 2H).

CONCLUSION

Ion mobility capabilities with the SYNAPT HDMS System offer an additional fourth dimension of information in the analysis of very complex samples by UPLC/MS. The DriftScope Mobility Environment Software is an essential tool to visualize the data and a serves as a very powerful aid to extract the information required, allowing only relevant drug-related information to be extracted. IMS technology could significantly accelerate the identification of metabolite peaks in cold *in vivo* samples by allowing scientists to obtain a more representative *in vivo* metabolism picture earlier on during drug discovery, where radiolabeled material is usually not available.

Acknowledgement

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A HIGH THROUGHPUT SCREENING METHODOLOGY FOR REACTIVE METABOLITE DETECTION AND IDENTIFICATION USING AN LC/MS/MS DATA-INDEPENDENT STRATEGY

Jose Castro-Perez, Kate Yu, John Shockcor, Henry Shion, Emma Marsden-Edwards
Waters Corporation, Milford, MA, U.S.

INTRODUCTION

The identification of metabolites, whether from *in vitro* or *in vivo* studies, is an ongoing challenge for drug discovery and development. Metabolite identification typically uses an array of chromatographic and mass spectrometric methods, and may require multiple injections of the same sample. This is to ensure that enough information has been collected to detect all metabolites and to have sufficient fragmentation information available to elucidate structures.

We describe in this application note a workflow that enables the collection of both parent and fragment information from a single injection (Figure 1). This MS^E data acquisition uses two interwoven scan functions: the first (low collision energy, or low CE) function contains data from the intact metabolites, and the second (high collision energy, or high CE) function contains data from the fragment ions.

The high throughput screening (HTS) analysis was performed using ACQUITY UPLC® and SYNAPT™ Mass Spectrometry systems. The resulting LC/MS data were obtained with mass accuracies typically in the sub-2 ppm range.

Since all data are collected in one run, post-acquisition processing of multiple fragment ions is possible. With this approach, the entire dataset is mined post-acquisition for specific metabolite masses, precursor and fragment ions, and neutral losses because all the necessary data has been collected simultaneously. Selectivity for biotransformation of the parent drug is achieved through exact mass measurement.

A variety of data processing algorithms have been used to extract metabolite information from these data.

From a single injection, it is possible to obtain neutral loss and precursor ion information with exact mass containing diagnostic losses for reactive metabolites for both neutral and precursor ions acquisitions. In turn, these diagnostic neutral losses and precursor ions may also be used for *in vitro* reactive metabolism screening, in conjunction with the low energy data, to confirm the presence of a reactive electrophile intermediate.

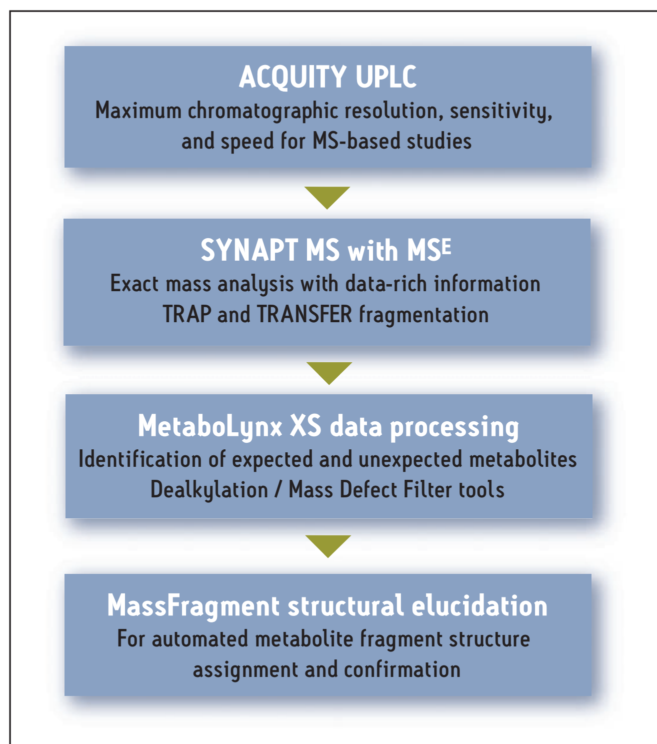


Figure 1. Workflow for metabolite identification using the ACQUITY UPLC/SYNAPT MS systems in TOF-MS^E mode.

We illustrate this data-independent UPLC®/MS reactive metabolite screening approach using samples from an incubation of Nefazodone human liver microsomes in the presence of glutathione (GSH).

EXPERIMENTAL

Samples

Nefazodone was incubated at 37 °C with rat liver microsomes at a final substrate concentration of 10 µM, in a Tris buffer adjusted to pH 7.4 containing the appropriate co-factors. GSH was added at a concentration of 10 mM to the microsomal incubation. The reaction was terminated after 90 minutes with two volumes of cold acetonitrile to one volume of sample. Then the sample was centrifuged at 13,000 RPM for 15 minutes and the supernatant was diluted in half with water +0.1 % formic acid. The diluted supernatant was injected directly to the UPLC/MS system for analysis.

Background on Nefazodone

Nefazodone is an antidepressant that was approved in the U.S. in late 1994. In spite of its therapeutic effects, there have been a number of cases – 55 cases of liver failure, 20 fatal, and another 39 cases of less severe liver failure – reported showing hepatobiliary dysfunction and cholestasis.[1]

LC conditions

LC system: Waters ACQUITY UPLC System
Column: ACQUITY UPLC BEH C_{18} Column 2.1 x 100 mm, 1.7 µm
Column temp.: 45 °C
Mobile phase A: 0.1 % formic acid
Mobile phase B: Acetonitrile
Flow rate: 0.6 mL/min
Gradient:

Time (min)	%A	%B	Curve
0.00	98	2	
8.00	40	60	6
9.50	0	100	6
12.50	98	2	1

Injection volume: 5 µL

MS conditions

Mass spectrometer: Waters SYNAPT MS System
MS scan range: 50 to 900 Da
Mode of operation: Positive and negative ion ESI
Lock mass: Leucine enkephalin at 200 pg/µL

Data processing

The MetaboLynx™ XS Application Manager, available for MassLynx™ Software, was used for MS^E data mining and detection of putative metabolites. MassFragment™ Software was used for structural elucidation of metabolites.

MS^E methodology[2, 3]

The SYNAPT MS System was operated in the MS^E data acquisition mode with a wide band RF mode in Q1, which allowed all ions to be transmitted from the source into the Triwave™ collision cells. The data were collected into a single data file with two functions. Function (1) Low CE acquisition (5 eV), which contained molecular ion information, and Function (2) High CE acquisition (using a 20 to 50 eV ramp), which contained all of the fragment ion information.[4]

The Triwave device provides the ability to induce fragmentation in two regions, Trap and Transfer (Figure 2), which results in enhanced fragmentation coverage across the mass range. For example, one can readily obtain valuable information at low m/z values with this approach that further assists the structural elucidation process.

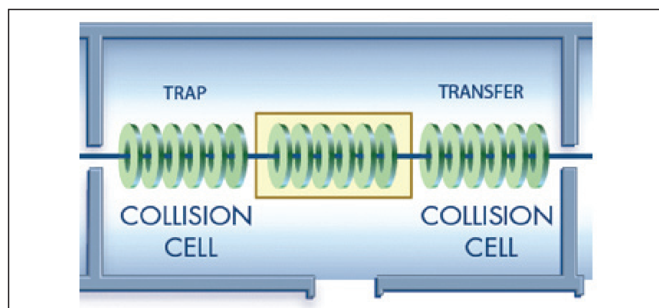


Figure 2. Schematic showing Triwave Trap and Transfer collision cells, which enable optimized fragmentation and structural coverage.

GSH trapping[5, 6]

Typical *in vitro* incubation in microsomes forced the reaction to form GSH adducts.

For positive ion mode, we monitored the loss of the pyroglutamic acid moiety m/z 129 (Figure 3), the loss of GSH m/z 307 for aliphatic and benzylic thioethers, and the loss of glutamic acid m/z 147 for thioesters.

For negative ion mode, we monitored the precursor ion at m/z 272 arising from the γ glutamyl-dehydroanalyl-glycine.

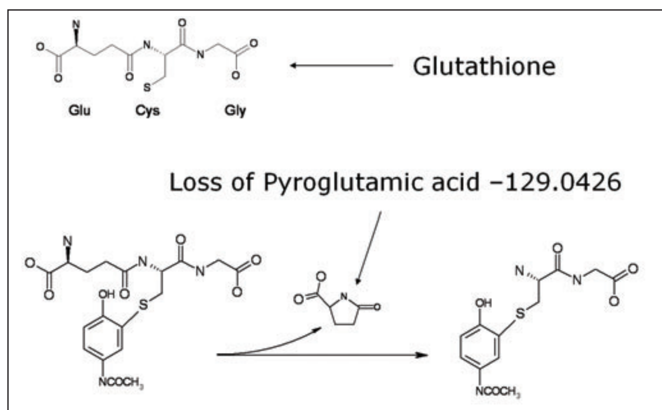


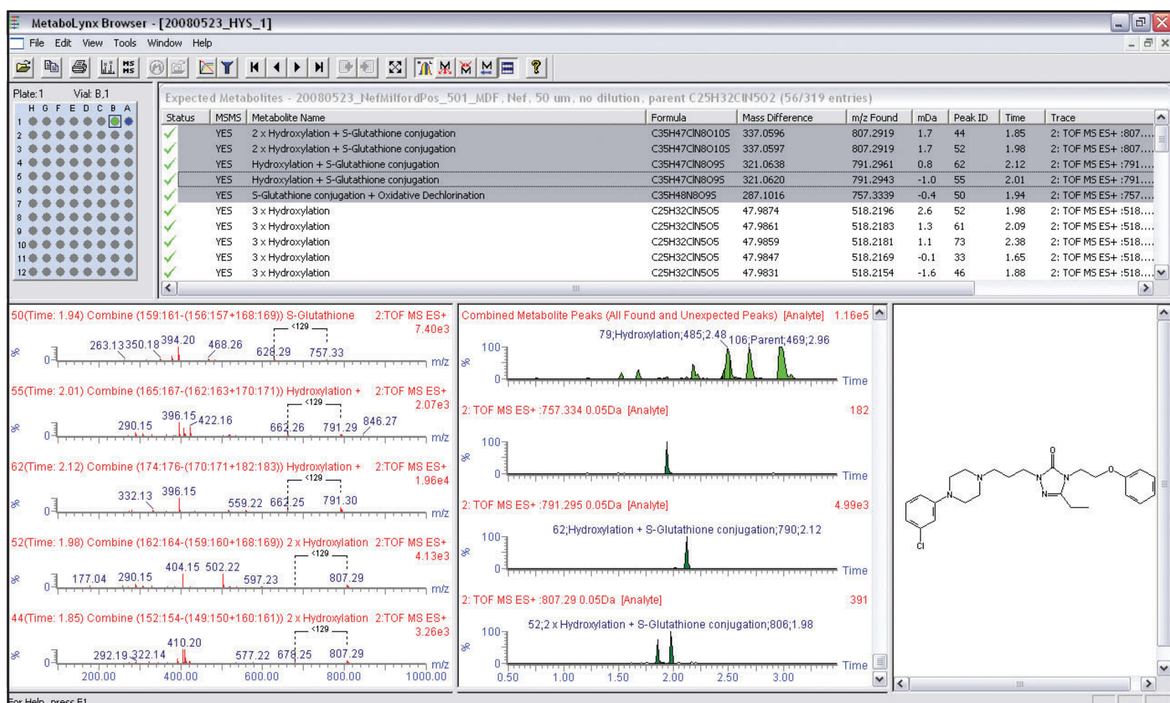
Figure 3. Neutral loss monitoring with the loss of the pyroglutamic acid moiety.

RESULTS

The data obtained by this approach were automatically processed with MetaboLynx XS. This automated metabolite identification software tool uses molecular structure to generate a list of dealkylations, which is then used to create an automated mass defect filter specific to the dealkylations and the additions of GSH plus any other phase I biotransformation combinations. This results in a much reduced list of false positives, helping to increase throughput and minimize time spent reviewing the data.

A total of five GSH adducts were detected in positive ion mode. These corresponded to m/z 757 (+O-Cl+GSH), 2x m/z 791 (+O+GSH), and 2x m/z 807 (+O2+GSH). The five GSH adducts were confirmed by reviewing the data in the low energy scan using exact mass (Figure 4).

Figure 4. MetaboLynx browser report showing all five GSH adducts as well as other biotransformations.



For further confirmation of these GSH adducts, the high energy data obtained in MS^E mode may also be used in parallel to further verify the existence and to localize the position of the GSH addition to the molecule. In this particular case, we searched for the signature neutral loss of the pyroglutamic acid m/z 129.0426, which is lost in the high energy mode (Figure 5).

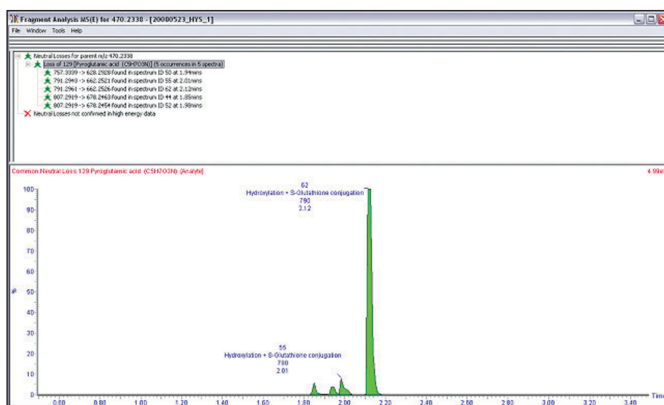


Figure 5. MetaboLynx Fragment Analysis report showing neutral loss of pyroglutamic acid for all five GSH adducts.

This approach is not confined to just searching one particular diagnostic loss since all the data are contained within the low and high energy acquisitions; we could potentially search for an unlimited number of neutral losses.

It is worth mentioning that, in some cases, the diagnostic neutral losses or precursor ions for GSH are not always generated. Even if this is the case, intact full-scan exact mass MS data is always available with this approach. This may not be the case with other techniques such as neutral loss scanning with a tandem quadrupole mass spectrometer, thus resulting in not detecting a potential GSH adduct. MS^E can be used to confirm the presence of a GSH adduct that does not follow the neutral loss rules and can be further verified in high energy mode.

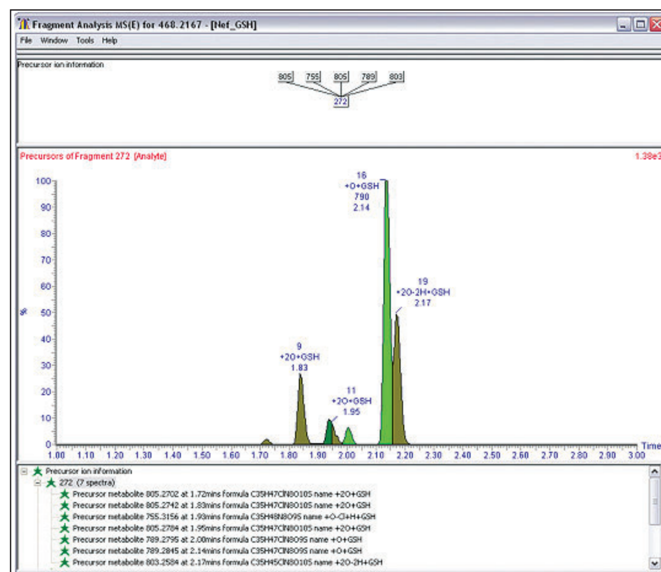


Figure 6. MetaboLynx negative ion precursor ion information for GSH adducts of Nefazodone.

Negative ion MS^E was also carried out and the GSH adducts were confirmed by extracting the diagnostic precursor ion of m/z 272 (Figure 6). In this example, we are showing the fragment data derived from the precursor ion of m/z 272 (the γ glutamyl-dehydroanalyl-glycine).

All the GSH adducts found in positive ion mode were confirmed, and two new adducts were confirmed in negative ion mode corresponding to m/z 803 (+2O-2H+GSH) and m/z 805 (+2O+GSH).

CONCLUSION

This application note demonstrates a streamlined, comprehensive, and generic workflow for metabolite identification, structural elucidation, and a specific search for GSH conjugations. It is possible to mine the data acquired in this fashion to extract information on multiple neutral losses or common precursor ions, which assist in identifying and localizing the sites of biotransformation.

From a single injection, we obtain data that would otherwise require numerous injections when utilizing traditional data-dependant analysis approaches such as those performed on tandem quadrupole or ion trap MS systems.

The novel software tools employed in this approach, MetaboLynx XS, allow the user to generate meaningful information from their samples in an automated manner with more confidence, thereby addressing one of the major bottlenecks in the drug discovery and development process.

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6. Dieckhaus et al. Chem Res Toxicol. 2005 ; 18 (4), 630-8.

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ENHANCED DUTY CYCLE ON A HYBRID QUADRUPOLE OA-TOF INSTRUMENT TO IMPROVE THE LIMIT OF DETECTION FOR REACTIVE METABOLITE SCREENING

Jose Castro-Perez, John Shockcor, Kate Yu, and Jeff Goshawk, Waters Corporation, Milford, MA, U.S.
Kevin Bateman, Merck Frosst, Kirkland, Canada

INTRODUCTION

Reactive metabolite screening in drug discovery is an integral part of the early screening process as it helps to detect possible new chemical entities (NCEs) that may undergo bioactivation. Such behavior can pose a threat to drug programs and is widely accepted to be a mechanism for xenobiotic-induced toxicity. Having the capability to detect reactive electrophiles earlier on in the discovery process allows pharmaceutical companies to re-optimize the compound in question and minimize the risk of toxicity at the development stage.

Typically, detection of reactive intermediates is carried out by LC/MS/MS experiments by using a combinations of precursor ion (PI) and neutral loss (NL) acquisitions with tandem quadrupole mass spectrometry.[1, 2] This technique proves to be selective because it is targeted towards specific losses of precursor ions as in the case of glutathione (GSH) trapping assays.

- For NL in positive ion mode, it is common to search for the loss of the pyroglutamic acid (m/z 129), for aliphatic and benzylic thioethers to monitor the loss of m/z 307, and for thioesters to monitor the loss of m/z 147.
- For PI, the ion at m/z 272 is monitored in negative ion mode corresponding to the γ glutamyl-dehydroanalyl-glycine.
- ESI positive ion mode will require multiple NL experiments to obtain maximum coverage for all types of NCEs due to the low duty cycle of scanning instruments. This may hinder detection of low-level GSH adducts.

The other significant factor is that most of the GSH adducts may manifest as the doubly-charged species $(M+2H)^{+2}$, which does not fragment during collision-induced dissociation (CID) to provide the appropriate NL of interest, but gives rise to singly-charged species. ESI negative ion acquisition may overcome some of the issues relating to doubly-charged formations and can become a more universal technique especially for GSH screening.

With this in mind, we have designed a novel approach that allows us to provide a highly sensitive methodology for GSH screening that can be further extended to other trapping assays such as cyano trapping or semicarbazide trapping.

In this application note, we describe a method that enables the collection of both parent and fragment information from a single injection using a hybrid quadrupole oa-TOF instrument, the SYNAPT™ High Definition MS (HDMS™) System, in negative electrospray ion mode. Data acquisition uses two interwoven functions where the first acquisition function collects information about the intact GSH metabolites, and the second acquisition function collects fragment ions, with an enhanced duty cycle operation for both acquisition modes. This increases the signal for diagnostic fragments of interest that notes the presence of the GSH adduct in both low and high energy acquisitions.

METHODS

Samples

Nefazodone was incubated with human liver microsomes at 10 µM at 37 °C, in a solution of 50 mM Tris buffer adjusted to pH 7.4 containing the NADPDH regenerating system. GSH was added at a concentration of 5mM to the microsomal incubation. The reaction was terminated after 60 min with 1 volume of cold acetonitrile to 1 volume of sample. Then the sample was centrifuged at 13,000 RPM for 15 min and the supernatant was injected directly to the UPLC®/TOF-MS system for analysis.

LC/MS/MS methodology

LC system:	Waters ACQUITY UPLC® System
Column:	ACQUITY UPLC HSS T3 C_{18} Column 2.1 x 100 mm I.D., 1.7 µm
Column Temp.:	45 °C
Mobile phase A:	5 mM Ammonium acetate, pH 5
Mobile phase B:	Acetonitrile
Flow rate:	0.6 mL/min
Gradient:	95% A to 25% A in 5 min, ramp 25 to 5% A in 1 min before returning to 95% A for re-equilibration
Injection volume:	10 µL
MS system:	Waters SYNAPT HDMS System
MS scan range:	50-900 Da
Mode of Operation:	Negative ion mode ESI
Lock Mass:	Leucine Enkephalin at 200 pg/mL

MS^E methodology[3,4]

The SYNAPT HDMS System was operated in parallel data-acquisition mode with a wide band RF mode in Q1 (Figure 1). This allows all ions to enter into the collision cell without pre-filtering. This enabled us to perform one single injection in which data was collected under one single data file with two functions. The first function, low energy acquisition (5eV) contains the intact compounds, and the second function, high energy or MS^E acquisition, uses the Triwave™ Technology with Trap (10 eV to 25 eV ramp) and Transfer (20 eV) regions that contain all of the fragmented ions.[5]

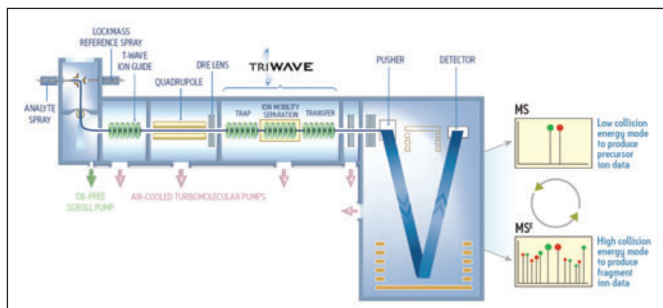

Figure 1. Schematic showing the MS^E methodology.

Enhanced Duty Cycle (EDC) methodology

In this mode of operation, the duty cycle of the mass spectrometer is increased by pre-defining the masses of interest. This provides a significant increase in sensitivity and limit of detection. This is achieved by utilizing the Triwave collision cell technology to shape the ion beam into discrete ion packets. These ion packets are then released from the collision cell at certain time intervals with the pusher of the oa-TOF, synchronized to operate as the ion of interest enters the extraction region (Figure 2). EDC may be operated in full scan MS or MS/MS mode.

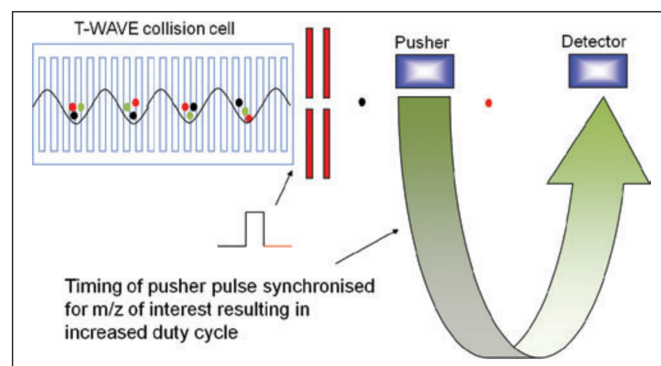

Figure 2. Schematic showing the mass spectrometer's EDC mode.

Processing software

The MetaboLynx™ Application Manager was used for the Enhanced Duty Cycle MS^E peak detection of putative metabolites. MassFragment™ Software was used for the structure elucidation step.

LC/MS/MS methodology for GSH reactive metabolite screening

For GSH trapping,[1,2] typical *in vitro* incubation in microsomes forces the reaction to form GSH adducts.

For positive ion mode:

- Monitor the loss of the pyroglutamic acid moiety m/z 129 (Figure 4)
- Monitor the loss of GSH m/z 307 for aliphatic and benzylic thioethers
- Monitor the loss of glutamic acid m/z 147 for thioesters

For negative ion mode:

- Monitor the precursor ion at m/z 272 arising from the γ glutamyl-dehydroanalyl-glycine.

Other GSH fragments may also be used (Figure 3).

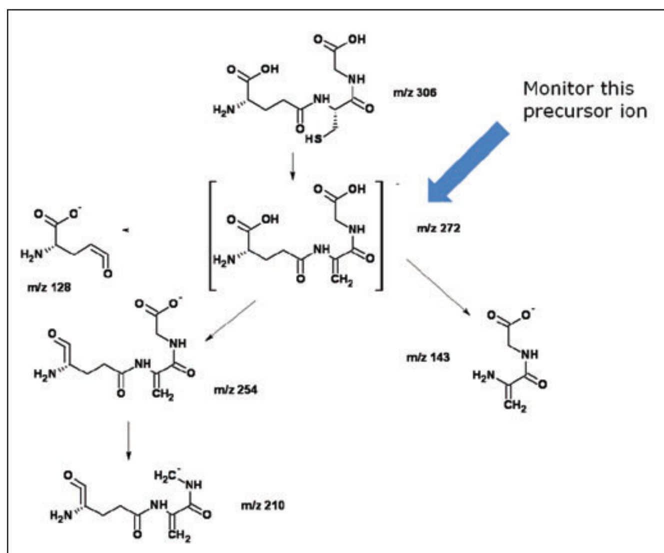

Figure 3. Negative ion ESI-CID fragmentation pathway for GSH.

RESULTS

The advantage of MS^E is that parallel information from the low and high energy is obtained in one single injection in an unbiased way. Figure 4 shows an example for one of the nefazodone O+ GSH adducts. Figure 5 shows that the spectra for the high and low energy is obtained in parallel.

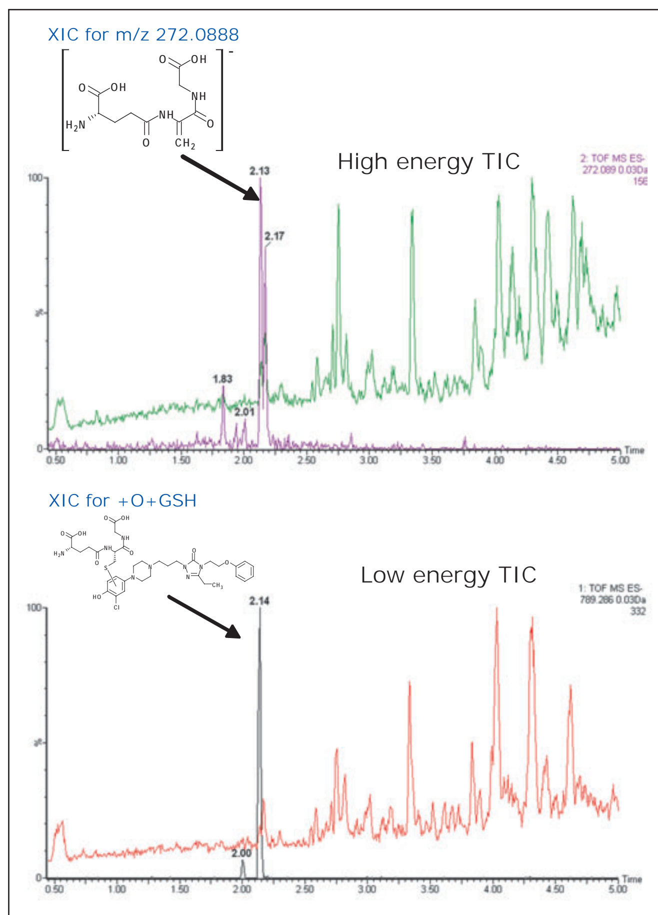

Figure 4. Low and high energy data for nefazodone in negative ion mode.

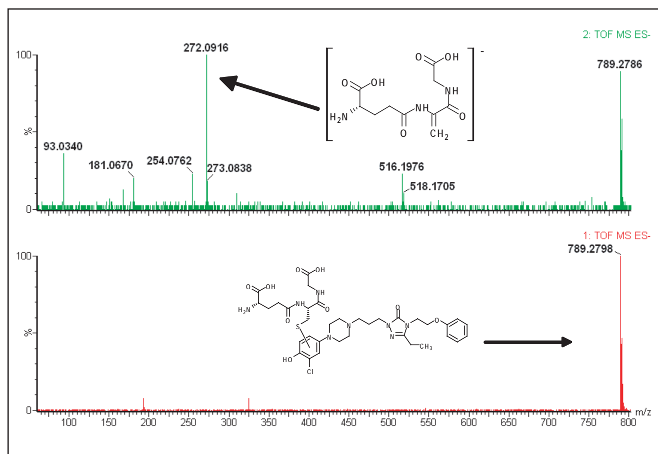

Figure 5. Low and high energy spectra in negative ion for the +O+GSH metabolite of nefazodone.

Even though we were searching for the specific diagnostic precursor ion of γ glutamyl-dehydroanalyl-glycine m/z 272 in negative ion mode, it is also possible to search for the corresponding neutral losses in positive ion mode. Because we see all of the fragment ion spectra in the high energy mode, it is possible to search for all losses or precursor ions of interest.

In this example, the mass at m/z 789.2798 from the low energy spectrum corresponds to the O+GSH metabolite of nefazodone.

From the same injection, the high energy spectra for the same mass at m/z 789.2789 gives rise to the diagnostic fragment ion of interest, which will help us to localize the GSH adduct and then confirm it in the low energy trace using exact mass.

The corresponding information was interrogated automatically with MetaboLynx, which was set up specifically to search for GSH adducts. This resulted in the detection of seven GSH adducts (Figure 6).

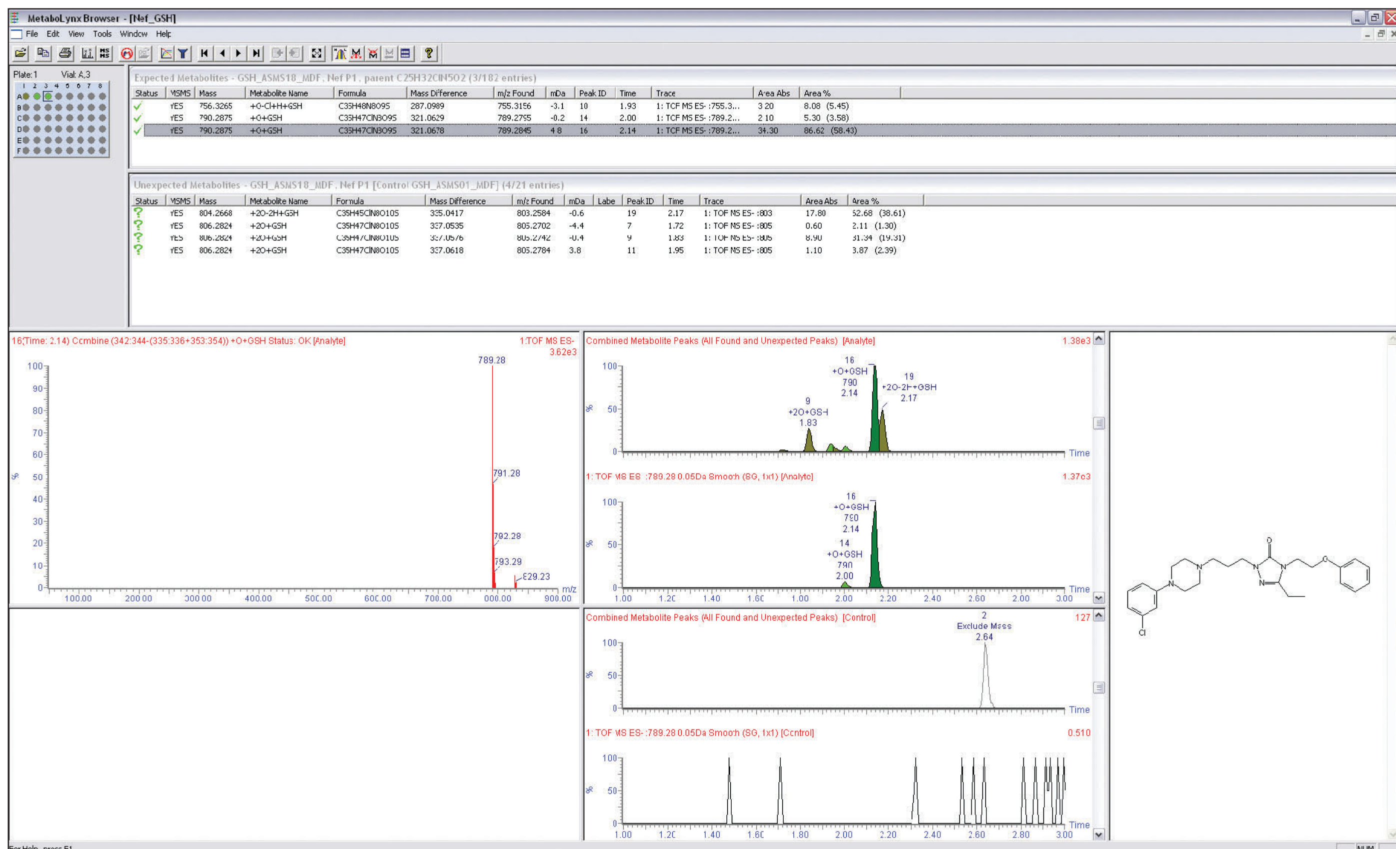

Figure 6. MetaboLynx browser showing all seven GSH adducts for nefazodone in the low energy mode.

Once the metabolites were detected in the low energy mode, this was followed by confirmation and alignment with the high energy data by monitoring the fragment ion at m/z 272, γ glutamyl-dehydroanalyl-glycine (Figure 7).

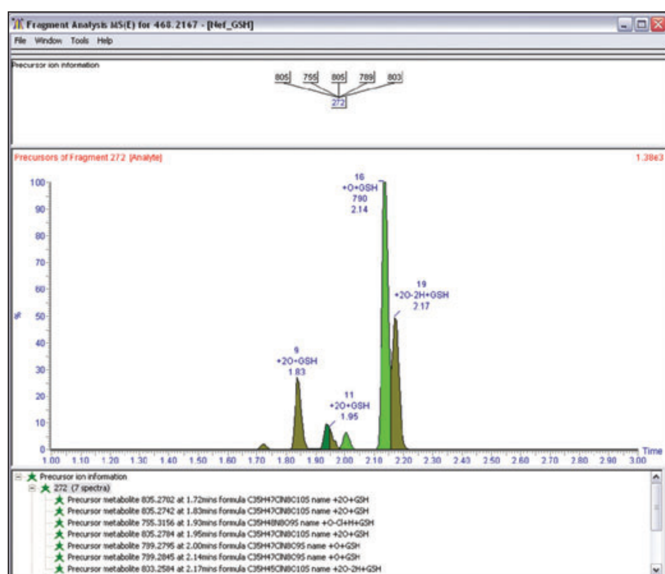

Figure 7. High energy data for all GSH adducts of nefazodone showing the precursor ion information for m/z 272, γ glutamyl-dehydroanalyl-glycine.

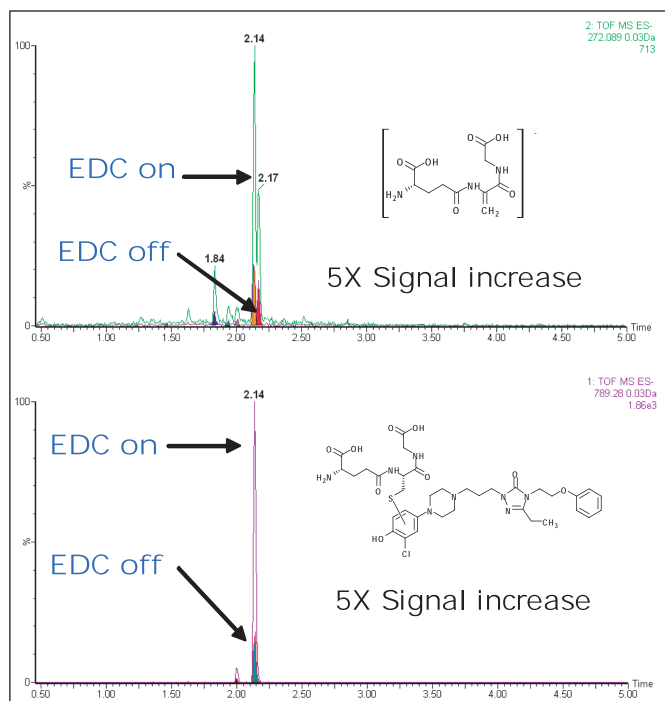

Figure 8. EDC on/off comparison for the +O+GSH metabolite and the fragment ion γ glutamyl-dehydroanalyl-glycine.

The use of the EDC function (Figure 8) in the low and high energy acquisition modes allowed us to obtain a much higher signal increase – up to five times higher than the normal mode of operation. Since in most cases GSH tend to be found at lower levels this enhances the likelihood of detection.

CONCLUSION

By combining ACQUITY UPLC and SYNAPT HDMS System technologies, the selectivity and superior sensitivity of using MS^E and EDC methodologies in parallel makes this analytical strategy very powerful for the detection of GSH adducts. MetaboLynx automates the process of GSH detection and interpretation.

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4. Bateman KP, Castro-Perez J, Wrona M, Shockcor JP, Yu K, Oballa R, Nicoll-Griffith DA. Rapid Communications in Mass Spectrometry. 2007; 21 (9): 1485-96.
5. The traveling wave device described here is silimar to that described by Kirchner in U.S. Patent 5,206,506 (1993).

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LOCALIZING DIAZEPAM AND ITS METABOLITE IN RAT BRAIN TISSUE BY IMAGING MASS SPECTROMETRY USING MALDI Q-TOF PREMIER MS

Jose Castro-Perez[1], Kate Yu[1], John Shockcor[1], Henry Shion[2], Tasneem Bahrainwala[2], Hidefumi Kaji[3], Yasuhiro Yamada[3]
[1] Waters Corporation, Milford, MA, U.S. [2] Waters Corporation, Beverly, MA, U.S. [3] Tanabe Seiyaku Co., LTD., Saitama, Japan

INTRODUCTION

There is increasing interest in the analysis of spatial distribution of small molecules in tissues for drug discovery, disease diagnosis, or biomarker discovery. Localization of the dosed drug and its metabolites are critical information for understanding the mechanism of target-organ toxicity.

Matrix-assisted laser desorption ionization (MALDI) is a sensitive solid-sampling and soft-ionization technique with extensive applications for the analysis of both large and small molecules. The MALDI mass spectrometry (MS) signal can be easily obtained directly from tissue sections.[1] The resulting three-dimensional image becomes very useful for the investigation of localization of dosed drug and its metabolites in tissue.

MALDI imaging provides an alternative to whole-body autoradiography in that there is no need to use a radiolabel to trace the drug and its possible metabolites throughout the organ of interest. This means that substantial savings are made, and, as a consequence, scientists can conduct an imaging experiment in targeted organs earlier on in the discovery process without the need of having a synthesized, radiolabeled new chemical entity.

Combining MALDI with quadrupole time-of-flight (TOF) MS, utilizing the Waters MALDI Q-Tof Premier™ Mass Spectrometer, offers excellent sensitivity and selectivity for these tissue imaging experiments.

In this application note, we use diazepam as an example to demonstrate the utility of MALDI TOF MS in this application area. After diazepam was intravenously administered to rats, a study using sliced rat brain tissues was performed. Results obtained showed clear localization of the parent drug and its metabolite.

Therefore, MALDI TOF MS proved sensitive, specific, and highly amenable to the image analysis of traditional small molecule drug candidates directly in tissues.

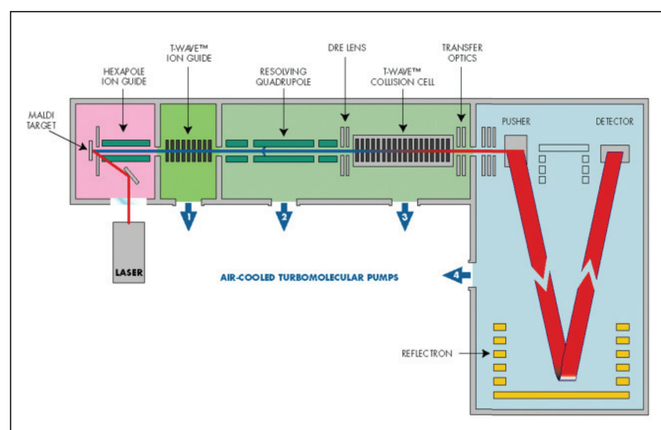

Figure 1. Schematic of the MALDI Q-Tof Premier.

EXPERIMENTAL

Sample preparation

Diazepam was intravenously administered to seven-week-old male Sprague-Dawley rats at three doses of 100, 30, and 10 mg/kg. Tissue samples were taken five minutes after administration. The isolated brain from the control and dosed rats were frozen by dry ice and embedded in the Tissue-Tek O.C.T. compounds (Sakura Finetek Japan, Tokyo). The tissue was sliced using a Cryostat (Leica CM-3050, Leica Microsystems) at a tissue thickness of 10 micro-m at 18 °C. The slices were mounted onto microscope plates.

The MALDI matrix used was α-cyano-4-hydroxycinnamic acid at 15 mg/mL in 50/50 acetonitrile/water (0.1% TFA). A TLC sprayer was used to deposit 15 layers of matrix onto the tissue.

MS conditions

Mass spectrometer:	Waters MALDI Q-Tof Premier
Mass range:	50 to 300 m/z
Laser type:	Nd: YAG
Repetition rate:	200 Hz
Collision energy:	25 eV
Gas and collision gas pressure:	Argon (5.30 x 10^{-3} mBar)
Data acquisition mode:	ESI+ MS/MS with EDC

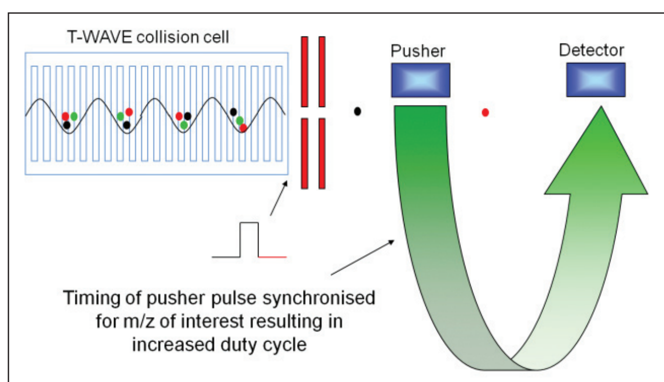

Figure 2. EDC set-up for enhanced MS/MS sensitivity.

RESULTS

The concentrations of diazepam and its metabolite, desmethyl diazepam, in rat brain after dosing have been previously determined by LC/MS/MS. The results are shown below.

Dose	Diazepam	Desmethyl Diazepam
10 mg/kg	20.2 μg/g	0.166 μg/g
30 mg/kg	50.5 μg/g	0.595 μg/g
100 mg/kg	266 μg/g	0.080 μg/g

The fragment ions selected for the tissue image were m/z 154 (diazepam) and m/z 140 (N-desmethyl diazepam). The use of EDC allowed the selected daughter ions at m/z 154 (diazepam) and m/z 140 (desmethyl diazepam) to be synchronized with the pusher, allowing an increased duty cycle increasing the signal up to five times more than using standard MS/MS conditions.

Figure 3 shows the tissue imaging of the diazepam (m/z 154) from the 100 mg/kg dosing, with the MALDI image on the left side and the tissue picture on the right side.

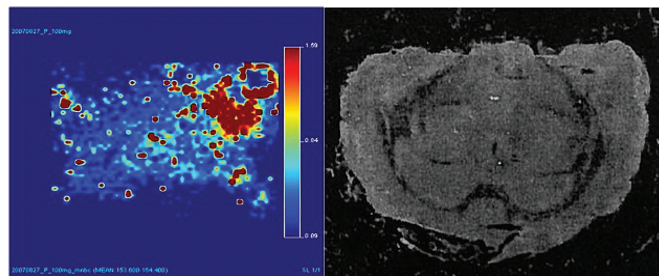

Figure 3. Rat brain tissue image for the 100 mg/kg dose.

The 100 mg/kg imaging showed an area of increased concentration on the top right corner of the brain with sparse spots of the drug in the tissue. This last finding was confirmed by autopsy of the animal, as the high dose lead to the death of the animal, with cerebral hemorrhage leading to the burst of blood vessels and leakage of the drug in the brain. As a result, no metabolite imaging was obtained for this dosage level.

Figure 4 shows the images corresponding to the 30 mg/kg dose. Figure 4A shows the image belonging to the parent drug. At this dosage, the drug has migrated to the lower right part (different distribution compared with the 100 mg/kg dose) with no sign of drug leakage through the vessels; the animal showed no sign of acute toxicity. Figure 4B shows imaging of the metabolite, N-desmethyl diazepam. The localization of the metabolite was not as confined to one region as the drug but more delocalized throughout the entire tissue.

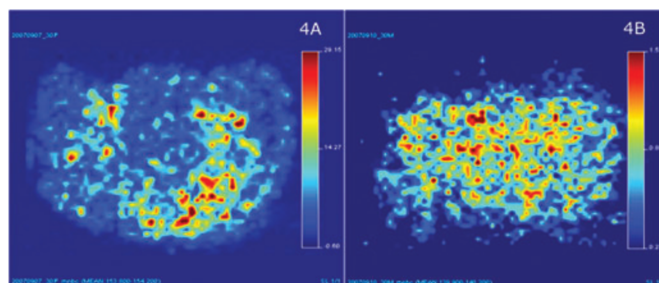

Figure 4. Rat brain tissue images for the 30 mg/kg dose.

Figure 5 shows the images corresponding to the 10 mg/kg dose. Figure 5A shows the image corresponding to diazepam. At this dose level, the localization of the parent drug was in the upper central part of the brain. Figure 5B shows the image corresponding to N-desmethyl diazepam. Similar to the 30 mg/kg dose, the metabolite was delocalized throughout the tissue, only at a lower concentration level.

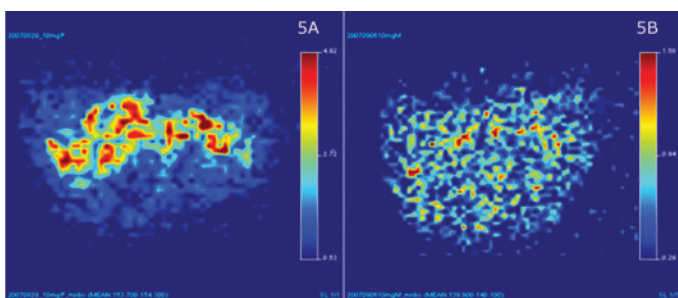

Figure 5. Rat brain tissue images for the 10 mg/kg dose.

DISCUSSION

The localization of the drug (diazepam) was noticeably different for all the concentrations analyzed. An explanation for this may be rationalized by the fact that since the blood-brain barrier penetration speed of diazepam is very fast, it is thought that localization immediately after administration is dependent on blood flow rate.

In other words, since the blood flow-dependent distribution is seen immediately after administration of diazepam, the distribution of high concentrations appears in the region where the blood flow rate is fast, and the distribution of low concentration is shown in the region where blood flow rate is slow.

In addition, it is reported that flow rate varies within regions of the brain, and the speed of fast regions is about five times that of the slow region. The reason that localization of the distribution for the N-desmethyl diazepam metabolite was not seen is that the penetration speed of blood brain barrier was slower than the parent drug.

CONCLUSION

MALDI TOF MS using the MALDI Q-Tof Premier Mass Spectrometer proved sensitive, specific image analysis of traditional small molecule drug candidates directly in tissues. MALDI imaging is a powerful technique used to visualize the localization of drug and metabolite in biological tissues. This particular approach, using EDC, provided enough sensitivity to monitor the drug and metabolite at low levels.

Reference

1. Stoeckli M, Chaurand P, Hallahan D, Caprioli R. Nature Med. 2001; 7 (4), 493-6.

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HIGH THROUGHPUT METABOLITE SCREENING AND SIMULTANEOUS DETERMINATION OF METABOLIC STABILITY USING METABOLYNX

Kate Yu, Jose Castro-Perez, and Robert S. Plumb
Waters Corporation, Milford, MA, U.S.

INTRODUCTION

In modern drug discovery, data obtained from *in vitro* metabolism studies are used in a prospective manner to choose lead drug candidates. A promising drug candidate should possess commercially acceptable pharmacokinetic (PK) properties. Early metabolic information is useful to guide the decision-making process for structural modifications to achieve the desired PK properties. In addition, the ability to evaluate *in vitro* metabolic stability in human liver microsomes is useful for predicting the clearance and half-life in humans of new chemical entities.

For discovery metabolism studies, throughput remains one of the most critical factors to consider during method development. Metabolism data should be generated and interpreted rapidly; fast turnaround times allow chemists to incorporate the data into synthesis of the next compound series.

Sample throughput in drug metabolism can be increased in two stages:

- Reducing instrument analysis time with Waters UltraPerformance LC® (UPLC®) technology, leveraging columns packed with sub-2 µm hybrid materials to retain the practicality and principles of HPLC separations while increasing analytical speed, sensitivity, and resolution
- Reducing the data processing and interpretation time with the help of MassLynx™ Software's MetaboLynx™ XS Application Manager

Data processing and interpretation has always been a major bottleneck for drug metabolism studies. MetaboLynx is a software tool that automates the process of peak detection and data interpretation. Results are presented in a browser with an easy-to-review format.

In addition, if the incubation was performed with multiple time points, MetaboLynx also automatically performs the metabolic stability calculation while performing the automated peak detection and data interpretation. This metabolic stability information is embedded within the MetaboLynx browser for easy review.

In this application note, we demonstrate the process of metabolite identification with a simultaneous determination of metabolic stability using MetaboLynx. The example used here is an *in vitro* microsome incubation of buspirone. All sample analyses were performed by using the ACQUITY UPLC® System with the Quattro Premier™ XE Mass Spectrometer (Figure 1).

Figure 1. The ACQUITY UPLC System with the Quattro Premier XE Mass Spectrometer for UPLC/MS analysis.

EXPERIMENTAL

In vitro microsome incubation

The parent drug buspirone was incubated with human and rat liver microsomes at a 100 μM level. The incubation was carried out at 37 °C, in a solution of 50 mM potassium phosphate adjusted to pH 7.4 containing the appropriate cofactors. The reaction was terminated with two volumes of cold acetonitrile to one volume of sample. The samples were stored frozen at -20 °C and diluted 1:2 ratio prior to UPLC/MS analysis.

UPLC conditions

LC System: Waters ACQUITY UPLC System
Column: ACQUITY UPLC BEH C_{18} 1.7 μm 2.1 x 150 mm
Column temp.: 90 °C
Mobile phase A: Water + 0.1% Formic acid
Mobile phase B: Acetonitrile + 0.1% Formic acid
Gradient:

Time (min)	Flow (mL/min)	%A	Curve
0.00	0.800	95.0	
5.25	0.800	30.0	6
5.70	0.800	0.0	1
8.00	0.800	95.0	1

MS conditions

Mass spectrometer: Waters Quattro Premier XE
Ionization mode: Electrospray positive
Capillary voltage: 3 kV
Cone voltage: 40V
Source temp.: 130 °C
Desolvation temp.: 470 °C
Acquisition mode: MS full scan

RESULTS AND DISCUSSION

A UPLC/MS full-scan experiment was performed for the incubated samples to obtain an initial screening of the metabolites. Figure 2 shows the extracted ion chromatograms of buspirone (m/z 386), hydroxybuspirone (m/z 402), and dihydroxybuspirone (m/z 418).

Previous reports[1, 2] have indicated that buspirone metabolites include six hydroxybuspirone and as many as eight dihydroxybuspirone. Results displayed in Figure 2 showed six hydroxybuspirone plus at least 13 dihydroxybuspirone. Further experiments are required to positively confirm the dihydroxybuspirones (beyond scope of this application note).

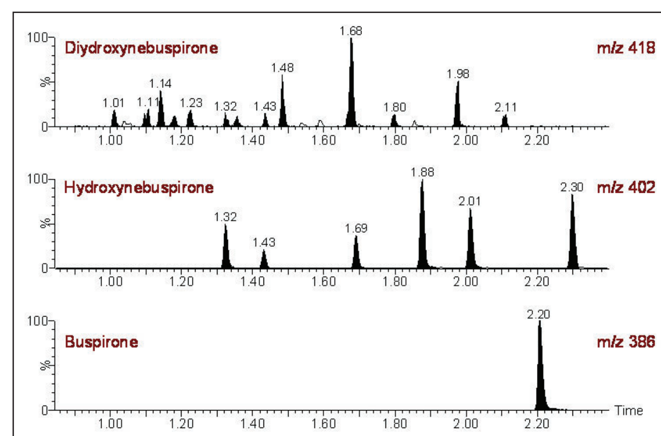

Figure 2. Selected ion chromatograms of buspirone and its metabolites obtained from the high temperature UPLC/MS full scan.

A complete report of the UPLC/MS full-scan experiments can be reviewed in the MetaboLynx browser, as shown in Figure 3.

In Figure 3, the upper-left corner displays the plate map with the location of samples injected. In this example, vial 1 (dark green) was the control sample, and vials 2 to 5 contain the incubated samples at incubation time points of 15 min, 30 min, 60 min, and 90 min.

The top panel displays the list of identified metabolites. The bottom-right panel displays chromatograms. Of the two chromatograms shown here, the upper chromatogram is the combined trace of all identified metabolites plus the parent drug. Each peak is

labeled with the metabolite's name, formula weight, and retention time. The lower chromatogram is the selected ion chromatogram of the hydroxybuspirone at m/z 402. The bottom-left panel shows the spectrum that corresponds to one of the hydroxylated metabolites of buspirone. This specific example shows the spectrum of the hydroxybuspirone with retention time of 1.7 min.

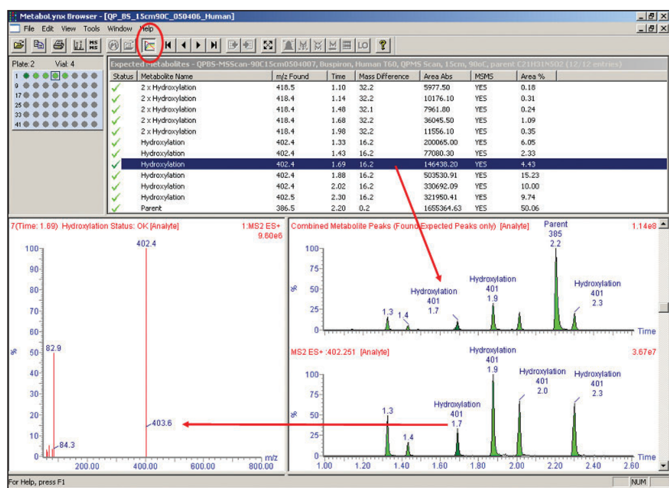

Figure 3. The MetaboLynx browser display of the LC/MS full-scan result for the buspirone incubated samples. The red circle highlights the metabolic stability button.

By clicking on the metabolic stability button (circled in red in Figure 3), a separate window is opened to display the metabolic stability results (Figure 4).

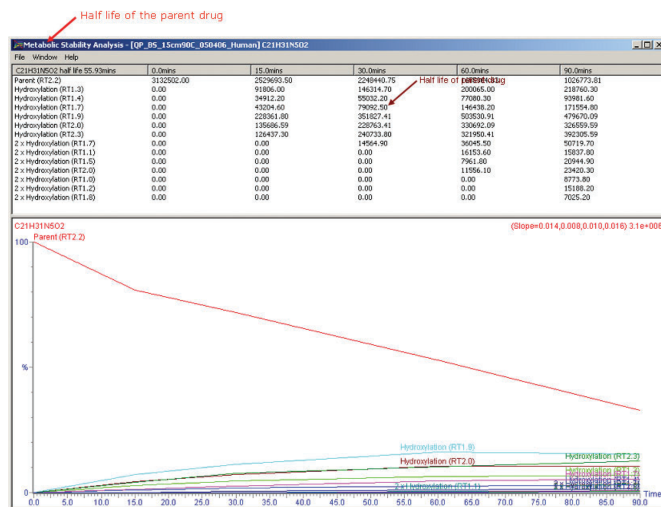

Figure 4. A screen capture of the rate of disappearance of buspirone and the rate of appearance of its metabolites.

The top panel in Figure 4 lists all of the identified metabolites, including the peak area of each metabolite at each time point. The half-life of the parent drug was calculated and shown on the top of the panel. In this example, the half-life for buspirone was 56 min. The bottom panel shows the plot of the disappearance for the parent drug as well as the appearance for metabolites.

CONCLUSION

We have demonstrated an entire information workflow that uses MetaboLynx to automatically perform data processing, result interpretation, as well as report generation. By performing a single UPLC/MS injection for each sample, we were able to obtain multiple levels of information. This includes the identification of potential metabolites, calculation of the metabolic stability such as half-life of the parent drug, as well as the rate of appearance of the metabolites.

As a result, the significant time-savings reaped by both the fast and complete UPLC/MS analysis as well as the resulting streamlined data analysis enables scientists to be more adept as they make decisions about the metabolic stability of subsequent compound synthesis in these high-throughput screening drug discovery activities.

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34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
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MASSFRAGMENT FOR STRUCTURAL ELUCIDATION IN METABOLITE IDENTIFICATION USING EXACT MASS MS

Kate Yu, Jose Castro-Perez, and John Shockcor
Waters Corporation, Milford, MA, U.S.

INTRODUCTION

Metabolite identification (Met ID) plays a crucial rule throughout the drug discovery and development process. The key analytical requirements for an effective Met ID study are: high chromatographic resolution with a fast turnaround time, metabolite identification with positive confirmation, and comprehensive structure elucidation.

Waters® UltraPerformance LC® (UPLC®) Technology has been gaining wide acceptance throughout the pharmaceutical industry since its first commercial instrument, the ACQUITY UPLC® System, was introduced in 2004. The system's ability to operate at 15,000 psi (1000 bar) backpressure enables the use of the sub-2 µm particle columns. The optimal linear velocity of this particle size extends far higher than that of the conventional 3 µm or 5 µm column packing. This higher linear velocity produces chromatographic separation with significantly enhanced resolution, speed, and sensitivity.

Waters SYNAPT™ MS TOF-based mass spectrometers are rapidly becoming the preferred analytical tool for Met ID studies. Its high full-scan sensitivity and exact mass measurement capability greatly enhance metabolite identification. In addition, the rich fragmentation information provided makes the task of localizing the site of biotransformation easier and more precise.

Finding metabolite "soft spots" is often a major bottleneck for the Met ID process. It is beneficial to have a tool that enables the scientist to take advantage of the information-rich data provided by the UPLC/SYNAPT MS system to rapidly find these soft spots.

This application note demonstrates the use of a UPLC/SYNAPT MS system employing the MS^E strategy for metabolite identification. Using the TOF MS^E, the LC/MS data from the low collision energy (CE) MS scan and high CE MS scan can be both obtained during a single UPLC injection.[1-2] The structural elucidation was easily performed with the aid of a software tool, MassFragment™, by directly importing the fragment ions from raw data file or from the MetaboLynx™ Application Manager's MS^E Fragment Analysis window.

METHODS

LC conditions

LC system:	Waters ACQUITY UPLC System
Column:	ACQUITY UPLC HSS T3 Column 2.1 x 100 mm, 1.7 µm
Column temp.:	45 °C
Flow rate:	600 µL/min
Mobile phase A:	Water + 0.1% Formic acid
Mobile phase B:	Acetonitrile
Gradient:	5% B to 95% B in 5 min, step to 100% and hold for 1 min
Run time:	8 min
Injection volume:	5 mL

MS conditions

MS system:	Waters SYNAPT MS
Ionization mode:	ESI Positive in TOF mode
Capillary voltage:	3000 V
Desolvation temp.:	450 °C
Desolvation gas:	800 L/Hr
Source temp.:	120 °C
Acquisition range:	50 to 800 m/z

TOF MS^E experiment

During an MS^E experiment, the mass spectrometer (MS) acquires full scan data in two separate functions. The first function applies low collision energy (CE) such as 5 eV. The second function applies high CE. There are three possible ways to set up the high CE scan. The collision energy can be set at a fixed value (e.g. 35 eV), or as a ramp (e.g. 20 eV to 40 eV), or as a profile (e.g. 15 eV, 30 eV, 45 eV).

The raw data file contains two chromatogram traces, one for each acquisition function. Other more biased approaches, such as traditional data-dependant experiments, may also be carried out with the SYNAPT MS. However, MS^E is an unbiased approach, which allows a much broader data coverage.

RESULTS

The data from the low CE MS acquisition typically offers parent ion information, while the data from the high CE MS scan offers fragment ion information. Figure 1 shows the comparison of the extracted ion chromatograms (XICs) and the MS spectra obtained from the two acquisitions during a single injection. The m/z 614 XIC at the low CE shows much higher intensity than that of the high CE spectrum. At higher CE, more fragmentation from the same parent ions occurred, resulting in the parent ion intensity being decreased.

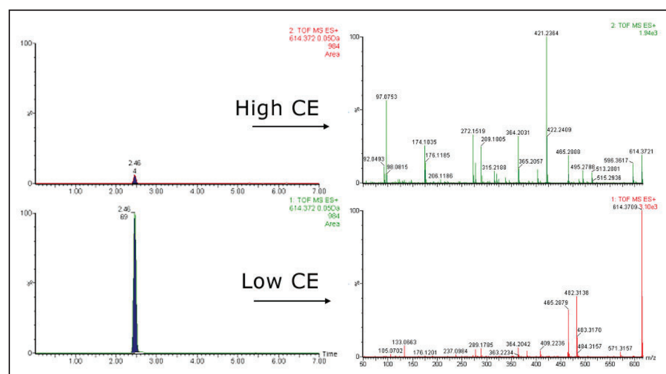

Figure 1. Extracted ion chromatograms and MS spectra obtained from low and high CE scans.

Figure 2 shows the chemical structure of indinavir. This structure can be easily created with any chemical drawing software and should be saved as a mol file that can be used by MassFragment during the elucidation step discussed in more detail later in this note.

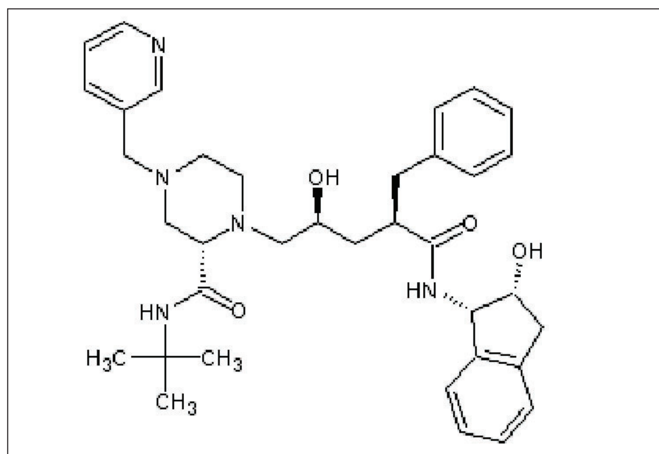

Figure 2. Chemical structure of indinavir ($C_{36}H_{47}N_5O_4$, MW 613.3628).

Once the MS^E data acquisition is complete, screening metabolites from the low CE data as well as the alignment of the fragment ion information from the high CE data is accomplished by the use of MetaboLynx, a MassLynx™ Software Application Manager. The MassFragment Software tool can be easily accessed from within MetaboLynx and helps to assign fragment ions displayed in the MS^E window in MetaboLynx.

MassFragment is also accessible from the Spectrum window in MassLynx (Figure 3). Upon selecting MassFragment, the dialog window opens requesting user's input of the parent drug .mol file (Figure 4).

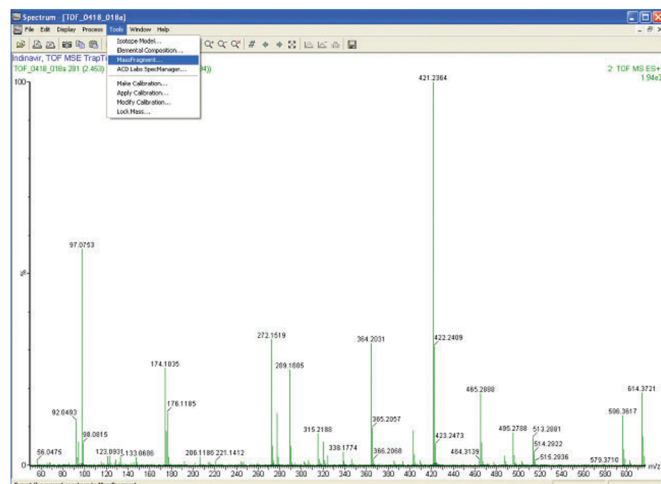

Figure 3. The MassFragment window in MetaboLynx displays the MS/MS information from an MS^E experiment.

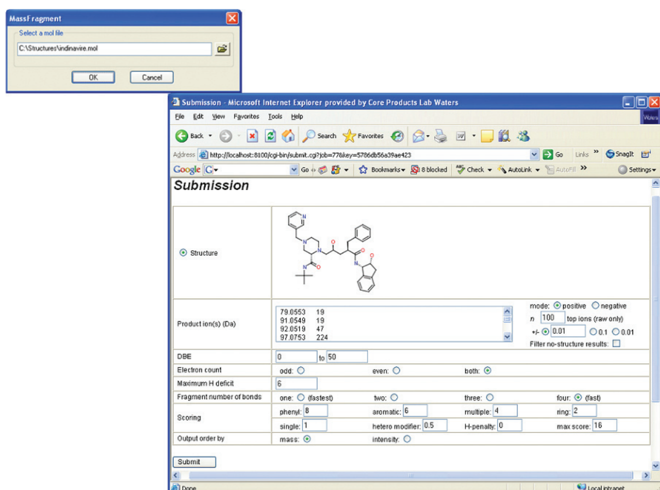

Figure 4. The mol file input window and the MassFragment interface.

Once the .mol file is selected, MassFragment is launched and a submission page is opened (Figure 4) with all the fragment ions automatically imported. The results window will display the proposed ion structures along with the exact mass errors and scores for each proposed structure. The scores are obtained based on the likelihood of breaking certain bonds and how well the results match with the exact mass generated. Therefore, the lower the score and the better the mass accuracy, the higher the confidence is in obtaining the correct structure for the fragment ion interrogated. The user can choose the fragment structures and print it into a .pdf file as a complete report (Figure 5).

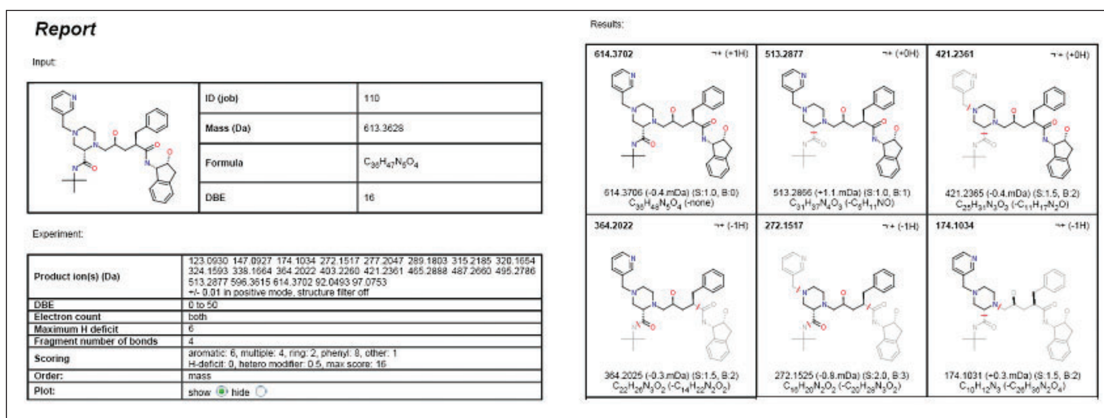

Figure 5. The PDF report generated by MassFragment.

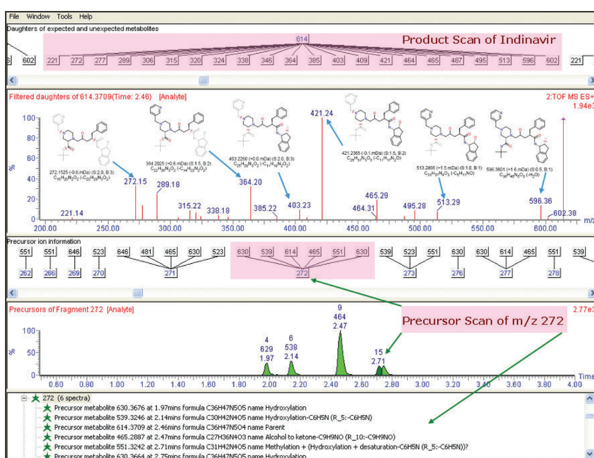

Figure 6. Structural elucidation for indinavir MS/MS fragment ions by MassFragment.

Figure 6 shows the final report from the MetaboLynx fragment analysis window. The fragment information obtained for the parent drug was assigned with fragments with the help of MassFragment. The lower half of the window shows the precursor ion scan from the m/z 272.1517, which is a signature fragment ion from the parent drug. This provides common fragment ion information with the parent and other drug related metabolites. This common precursor ion information helps to localize the position of the biotransformation in the molecule in a single injection.

The advantage of MS^E together with MassFragment is that it allows the user to obtain all this information (fragment ion, precursor ion, and neutral losses) from a single injection and organize the information for structure elucidation in a simple data viewer.

CONCLUSION

In this application note, we have demonstrated a strategy for the rapid approach to metabolite identification and structural elucidation using Waters SYNAPT MS TOF-based mass spectrometer operating in MS^E mode, with streamlined data analysis using the MetaboLynx and Mass Fragment Software tools.

Compared with the traditional data-dependant approach, which requires multiple injections for screening potential metabolites and performing structural elucidation with fragment ion information, this strategy offers enhanced productivity while providing superior data.

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AN INTELLIGENT UPLC/TOF-MS WORKFLOW FOR METABOLITE IDENTIFICATION USING A SOFTWARE TOOL INCLUDING C-HETEROATOM CLEAVAGES AND AUTOMATIC GENERATION OF MASS DEFECT FILTERS

Jose Castro-Perez, John Shockcor, Kate Yu, Henry Shion, Jeff Goshawk
Waters Corporation, Milford, MA, U.S. and Manchester, UK

Russell Mortishire-Smith
Johnson & Johnson Pharmaceutical Research and Development, Beerse, Belgium

Alastair Hill, Michael Hartshorn
Dotmatics Ltd, Herts, UK

INTRODUCTION

The major bottleneck in metabolite identification is data processing, because it is still mainly a manual process. The biggest challenge for automating data processing is managing the large number of false positives that may be generated.

A powerful software tool to help with the removal of these false positives is the Mass Defect Filter (MDF).[1,2] Optimizing the MDF on a compound-specific basis is an important step, since different compounds will give rise to different metabolic cleavages and may not exhibit a predictable fixed linear range of mass defect, such as drugs containing S, Cl, or Br.

We have previously presented a proof of concept approach that uses a simple algorithm that can quickly generate an intelligent compound-specific mass defect filter.[3]

In this work, we present the integration of this algorithm into a fully-featured metabolite identification workflow, utilizing the ACQUITY UPLC® System for chromatographic separation and the SYNAPT™ HDMS™ System for ion-mobility mass spectrometry along with MetaboLynx™ and MassFragment™ software for automated data processing (Figure 1). This allows us to query a particular drug up front to generate a list of C-heteroatom and heteroatom-heteroatom cleavages. This information is used to generate an automatic filter with adjustable limits depending upon whether Phase 1 or 2 metabolites are selected.

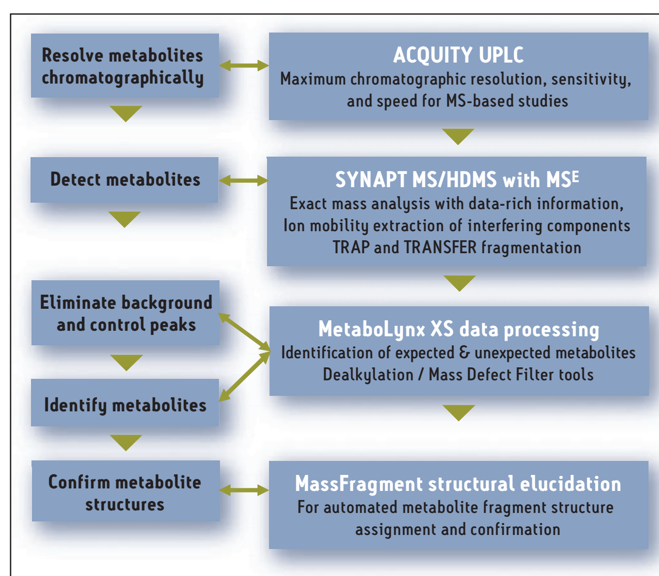

Figure 1. UPLC/MS^E metabolite identification workflow.

METHOD

Samples

Nefazodone and indinavir were incubated with rat liver microsomes at 10 µM at 37 °C, in a solution of 50 mM Tris buffer adjusted to pH 7.4 containing NADPH regenerating system and GSH at 5 µM. The reaction was terminated after 90 min with two volumes of cold acetonitrile to one volume of sample. Then the sample was centrifuged at 13,000 RPM for 15 min and the supernatant was diluted 1/2 with water +0.1 % formic acid. Finally, the supernatant was injected directly to the UPLC®/MS/MS system for analysis.

LC/MS Methodology

LC system:	Waters ACQUITY UPLC System
Column:	ACQUITY UPLC BEH C_{18} Column
	2.1 x 100 mm I.D., 1.7 µm
Column temp.:	45 °C
Mobile phase A:	0.1 % Formic acid
Mobile phase B:	Acetonitrile
Flow rate:	0.6 mL/min
Gradient:	98% A to 40% A in 8 min, ramp to 40 to 0% A in 1.5 min before returning to 98% A for re-equilibration
Injection volume:	5 µL
MS system:	Waters SYNAPT HDMS System
MS acquisition:	50 to 900 Da
Mode of operation:	Positive ion mode ESI
Lock mass:	Leucine Enkephalin at 200 pg/mL

Processing software

The MetaboLynx Application Manager, part of MassLynx™ Software, was used for MS^E data mining[4,5] and peak detection of putative metabolites (Figure 2). MassFragment was used for structure elucidation.

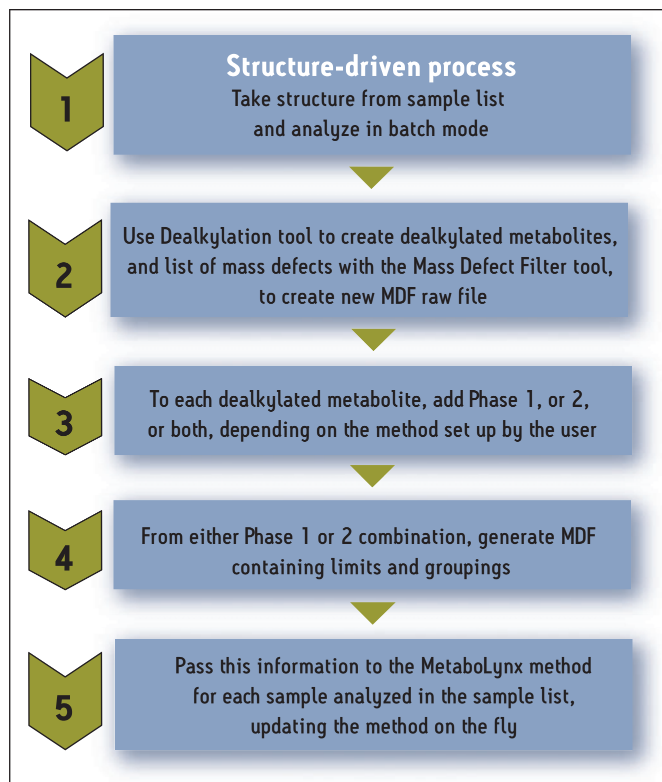

Figure 2. MetaboLynx's chemically-intelligent workflow.

Mass Detect Filter

The Mass Defect Filter (MDF) can be utilized to remove false positives and improve the metabolite identification workflow when combined with prior knowledge of the mass and its decimal places (elemental composition). The mass defect can either be positive (larger than the nominal mass) or negative (smaller than the nominal mass).

For example, the mass defect for H is +0.0078, with 1.0078 as its exact mass. The mass defect for OH is –0.0051, with 15.9949 as its exact mass.

This software applies a post-acquisition data filtering technique that is set based upon the mass defect of the parent drug and its metabolites. The rationale and assumption supporting MDF are that the majority of metabolites only have small shifts in their mass defect when comparing with the exact mass of the parent drug.

Applying the MDF technique allows users to capture and identify metabolites from complex matrices in a rapid and effective manner.

However, applying the MDF can be challenging and very much compound-specific. First, the mass defect may have rather large significant shifts when a dealkylation occurs for the parent drug prior to the formation of metabolites (Figure 3). Second, the mass defect shifts are not linear to their respective integer masses (Figure 4).

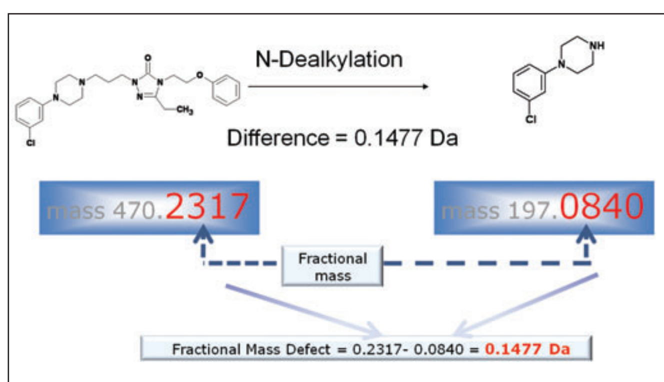

Figure 3. Mass defect shift after N-dealkylation occurred for nefazodone.

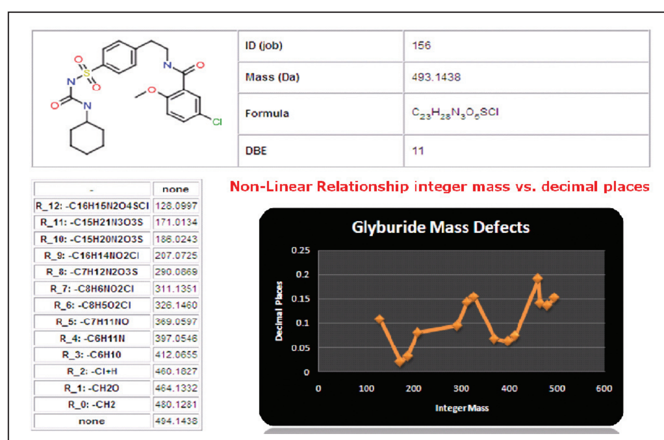

Figure 4. Mass defect shift after dealkylation occurred for glyburide.

MDF in MetaboLynx

In the UPLC/MS^E metabolite identification workflow shown in Figure 1, the Mass Defect Filter is applied at the initial stage during the MetaboLynx data processing. Once the user sets up the MetaboLynx method, the parent drug structure is first automatically transferred to the dealkylation tool to identify metabolic cleavages.

Upon identifying the major dealkylated fragments, their corresponding MDFs are automatically grouped with expected biotransformations. The MDFs are calculated based on their individual metabolic cleavages and biotransformation addition to each metabolic cleavage and parent.

Having done this, MetaboLynx can generate mass defect filtered chromatograms for control and dosed samples (.MDF files). The next step is for MetaboLynx to go through its regular metabolite identification procedure using the filtered chromatograms, with the aim of having a much more reduced list of unexpected metabolites.

RESULTS

Figure 5 shows how the mass defect filters are automatically set in MetaboLynx. Because of the large fluctuation of the mass defect as a result of dealkylation, multiple mass defect filters were automatically set for the metabolite identification.

Figures 5, 6, and 8 also show the automatic generation of metabolic cleavages for nefazodone and indinavir. Comparisons of the number of nefazodone and indinavir metabolites identified using linear-fixed MDF and intelligent MDF are shown in Figure 7 and Figure 9, respectively.

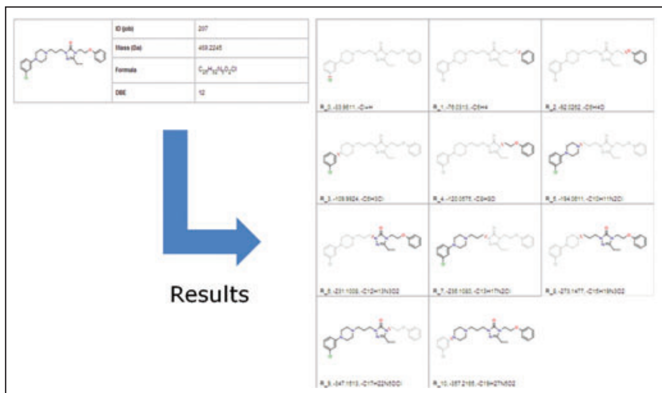

Figure 5. Automatic generation of metabolic cleavages for nefazodone.

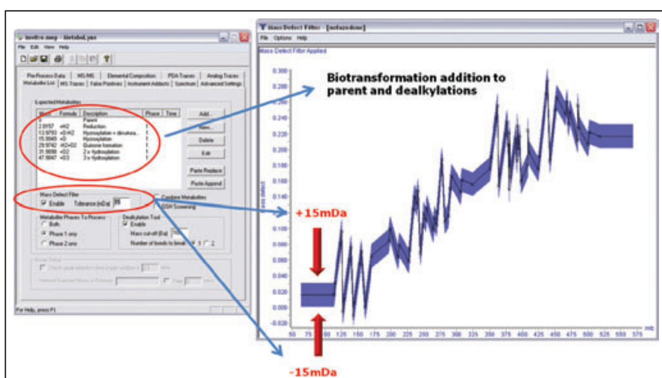

Figure 6. Automatic generation of metabolic cleavages and Mass Defect Filers for nefazodone.

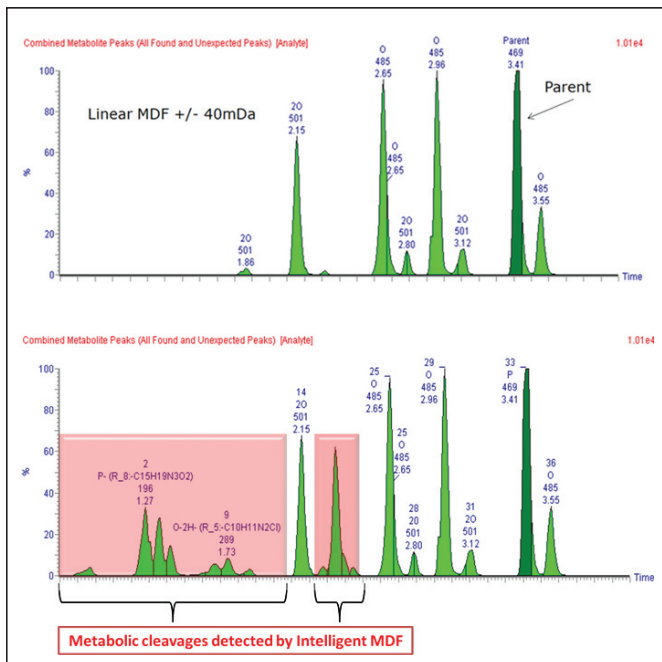

Figure 7. Nefazodone metabolites comparison using linear-fixed MDF (top) and intelligent MDF (bottom).

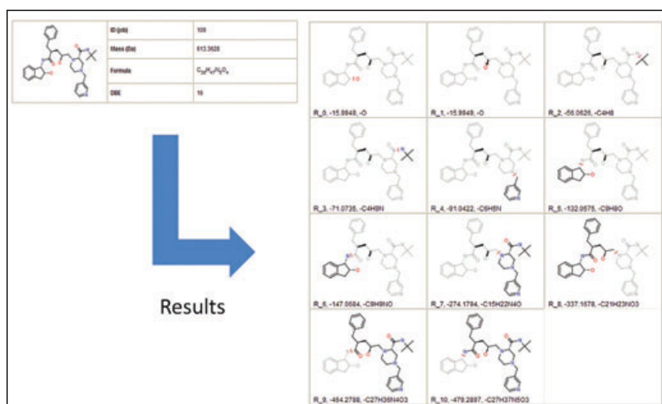

Figure 8. Automatic generation of metabolic cleavages for indinavir.

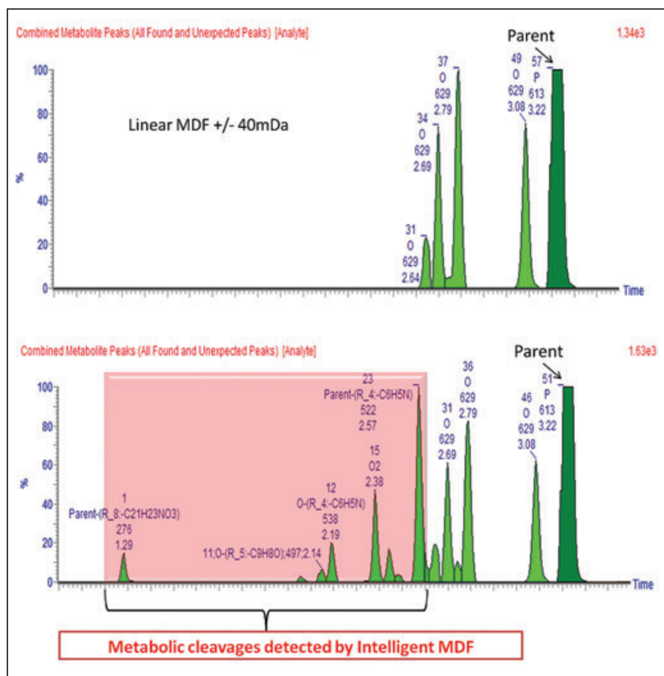

Figure 9. Indinavir metabolites comparison using linear-fixed MDF (top) and intelligent MDF (bottom).

CONCLUSION

With the use of MetaboLynx's dealkylation tool in combination with the Mass Defect Filter, we are able to rationalize the expected and unexpected metabolites more efficiently and minimize the number of false positives.

From this initial step it is possible to generate an extensive expected metabolite list on the fly. The unexpected metabolites, such as ring contractions or formations, are also possible to detect since we allow for a confidence limit window for the MDF, which will cover such biotransformations.

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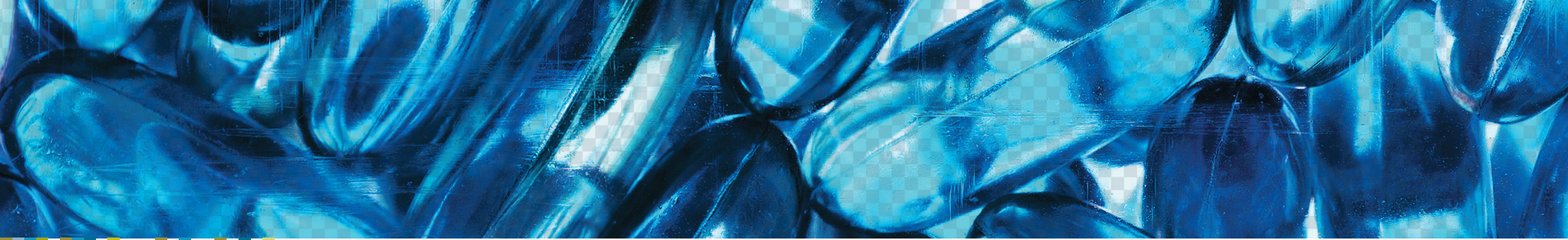

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